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<b>(21) International Application Number:</b> PCT/US00/03037 <b>(22) International Filing Date:</b> 4 February 2000 (04.02.00)  <b>(30) Priority Data:</b> 60/118,902                      5 February 1999 (05.02.99)      US 60/172,754                      20 December 1999 (20.12.99)      US  <b>(71) Applicant (for all designated States except US):</b> MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> BAI, Chang [CN/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).  <b>(74) Common Representative:</b> MERCK & CO., INC.; 126 Lincoln Avenue, Rahway, NJ 07065-0907 (US).		<b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> METHODS OF USE FOR DNA MOLECULES ENCODING M68, A TNF RECEPTOR-RELATED PROTEIN  <b>(57) Abstract</b>  <p>The present invention relates to human DNA molecules encoding M68, a tumor necrosis factor (TNF) receptor-related protein, recombinant vectors comprising DNA molecules encoding M68, recombinant host cells which contain a recombinant vector encoding M68, the M68 protein encoded by the DNA molecule, methods of identifying selective modulators of M68, and additional methods of use for the DNA encoding M68 and the expressed protein.</p>		

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## TITLE OF THE INVENTION

METHODS OF USE FOR DNA MOLECULES ENCODING M68,  
A TNF RECEPTOR-RELATED PROTEIN

## CROSS-REFERENCE TO RELATED APPLICATIONS

This provisional application is a continuation-in-part of U.S. Provisional Application Serial No.60/172,754, filed December 20, 1999, which is a continuation-in-part of U.S. Provisional Application Serial No. 60/118,902, filed February 5, 1999.

## STATEMENT REGARDING FEDERALLY-SPONSORED R&amp;D

Not Applicable

## REFERENCE TO MICROFICHE APPENDIX

Not Applicable

## FIELD OF THE INVENTION

The present invention relates to human DNA molecules encoding M68, a tumor necrosis factor (TNF) receptor-related protein, recombinant vectors comprising DNA molecules encoding M68, recombinant host cells which contain a recombinant vector encoding M68, the M68 protein encoded by the DNA molecule, methods of identifying selective modulators of M68, and additional methods of use for the DNA encoding M68 and the expressed protein.

## BACKGROUND OF THE INVENTION

Tumor necrosis factor (TNF) family and TNF receptor (TNFR) families comprise a group of molecules involved in the regulation of immune response, cell proliferation/differentiation, and cell death. It is known that various ligands (e.g., TNF- $\alpha$ , sTNF- $\alpha$ , OPGL, LT $\alpha$  (TNF- $\beta$ ), TRAIL, FasL, CD40L, CD30L, CD27L, 4-1BBL, LIGHT, etc.) and receptors (e.g., p55R, p75R, Fas, LT $\beta$ R, CD40, CD30, NGF-R, etc.) members of this family play an important role in apoptotic cell death (for a recent review, see Wallach, 1996, Cytokine & Growth Factor Reviews 7: 211-221).

PCT International Application WO 98/30694 discloses a cDNA molecule which comprises the same open reading frame as the DNA molecules encoding M68. The inventors suggest broad roles which the expressed protein may have, including roles in immune system-related disorders.

Pitti et al. (1998, *Nature* 396: 699-703) disclose a cDNA molecule which comprises the same open reading frame as the DNA molecule encoding M68. The authors refer to the expressed protein as decoy receptor 3 (DcR3) and show mRNA expression in fetal lung, brain and liver as well as adult spleen, colon and lung. The DcR3 gene was also shown to be amplified in various primary lung and colon tumors. The authors suggest a role which involves interaction with Fas ligand, a TNF- $\alpha$  related protein involved in apoptosis (see also Green, 1998, *Nature* 396: 629-630).

Osteoprotegerin (OPG) has been identified as a member of the TNFR family. This protein lacks a transmembrane domain and is a secreted protein which regulates bone metabolism, leading to the possibility that this protein is a natural inhibitor for its ligand and a possible candidate for use in treating bone diseases such as osteoporosis (Simonet et al., 1997, *Cell* 89(2):309-319).

PCT International Application WO 96/28546 discloses a cDNA molecule which encodes a putative TNF receptor-related protein.

PCT International Application WO 98/12344 discloses a cDNA molecule referred to as TR1 receptor, which shows homology to the type 2 TNF receptor.

PCT International Application WO 98/18824 discloses a cDNA molecule referred to as TR2 receptor, which shows homology to the type 2 TNF receptor.

PCT International Application WO 98/54201 discloses a cDNA molecule referred to as TR8 receptor, which shows homology to the type 2 TNF receptor.

It would be advantageous to identify additional members of the TNFR-like family which are expressed in a variety of tissue types and are involved in apoptosis-related diseases, such as cancer, inflammation, infections, autoimmune disease and osteoporosis. The present invention addresses and meets these needs by disclosing isolated nucleic acid molecules which express human M68, recombinant vectors which house this nucleic acid fragment, recombinant host cells which express human M68 and/or a biologically active equivalent, methods of identifying modulators of M68 and other uses for the nucleotide and amino acid sequence which define the respective gene and expressed protein, M68.

## SUMMARY OF THE INVENTION

The M68 protein is a member of a family of proteins which have roles in immune responses, cell death, cell proliferation and stimulation of cell differentiation, resulting in testing for potential use in the treatment of a variety of disease including cancer, inflammatory diseases, infections, autoimmune disease and osteoporosis and other bone disorders. Unlike most other TNFRs, M68 lacks a transmembrane domain and is a secreted factor. Thus it is predicted to function as a natural inhibitor for its ligand. The altered expression pattern in a multitude of tissues, as disclosed herein,

suggests that M68 may play a role in cancer by binding to its ligand and blocking apoptotic cell death induced by such a ligand. This anti-apoptotic role of M68 suggests that modulators of M68 will be useful in treatment of apoptosis-related diseases, including but not limited to various forms of cancer and various bone disorders, which are characterized by altered apoptotic activity.

Therefore, the present invention relates to methods of using DNA molecules which express human M68, as well as the expressed M68 ligand which are useful in identifying modulators of the M68, a ligand or ligand(s) of M68, or competing ligands or receptors. An antagonist of M68 will be useful to prevent or inhibit tumorigenesis, especially tumorigenesis effected by interaction of molecules involved in apoptosis in such cancers as cancers of the colon, esophagus, rectum and stomach. A compound which acts as an agonist of M68 activity will be useful in the treatment of diseases in which an excessive or inappropriate apoptosis was induced by its ligand.

The present invention also relates to use of the nucleic acid molecules disclosed herein for use in various diagnostic protocols to measure M68 expression and/or mutation in regard to various cancers, including but not limited to colon, esophagus, rectum and stomach cancer.

The present invention also relates to use of the nucleic acid molecules disclosed herein for use in mutation screening or gene amplification studies.

The present invention also relates to use of the nucleic acid molecules disclosed herein as gene therapy vehicles, wherein *in vivo* expression will result in increase levels of the M68 protein, which in turn will be useful in treating conditions involving atypical apoptosis.

The present invention relates to an isolated or purified nucleic acid molecule (polynucleotide) which encodes the TNFR-like protein, M68. The nucleic acid molecules of the present invention are substantially free from other nucleic acids. The present invention is in part exemplified by the isolated DNA molecule comprising the nucleotide sequence disclosed herein as SEQ ID NOs:1, 2 and 5.

The present invention also relates to isolated fragments or mutants of SEQ ID NOs:1, 2 and 5 which encode mRNA expressing a biologically active protein or protein fragment of M68. Any such biologically active fragment and/or mutant will encode either a protein or protein fragment which at least substantially mimics the pharmacological properties of a wild-type, M68 protein, including but not limited to the M68 receptor protein as set forth in SEQ ID NO:3. Any such polynucleotide includes but is not necessarily limited to nucleotide substitutions, deletions, additions, amino-terminal truncations and carboxy-terminal truncations such that these mutations encode mRNA which express a protein or protein fragment of diagnostic,

therapeutic or prophylactic use and would be useful for screening for agonists and/or antagonists for M68 function.

A preferred aspect of this portion of the present invention is disclosed in Figure 1A-B, a human cDNA molecule encoding M68 (SEQ ID NO:1).

Another preferred aspect of this portion of the present invention is disclosed herein and set forth as SEQ ID NO:2, a human cDNA molecule which comprises a different 5' UTR.

Another preferred aspect of this portion of the present invention is disclosed herein and set forth as SEQ ID NO:5, which comprises an isolated genomic clone encoding human M68. Especially preferred are non-coding regulatory regions of the genomic clone which are involved in regulation of M68 gene expression. These non-coding regions are useful in assays to identify regulatory element/factors for M68 transcription and thus to further identify reagents that modulate M68 expression. Such modulators will be especially useful to treat diseases where M68 expression is abnormal. It is shown herein that the overexpression of M68 in GI cancers is in fact independent of M68 gene amplification, which implicates *cis*-acting regulatory sequences and/or regulatory factors (such as proteinaceous *trans*-acting factors) in the control of this abnormal M68 expression level seen in various cancers.

The isolated nucleic acid molecules of the present invention may include a deoxyribonucleic acid molecule (DNA), such as genomic DNA and complementary DNA (cDNA), which may be single (coding or noncoding strand) or double stranded, as well as synthetic DNA, such as a synthesized, single stranded polynucleotide. The isolated nucleic acid molecule of the present invention may also include a ribonucleic acid molecule (RNA).

The present invention also relates to recombinant vectors and recombinant hosts, both prokaryotic and eukaryotic, which contain the substantially purified nucleic acid molecules disclosed throughout this specification.

A preferred aspect of the present invention is disclosed in Figure 2 and Figure 3 (set forth as SEQ ID NO:3) and especially the mature form disclosed in Figure 4 and as set forth as SEQ ID NO:4. The M68 protein is a TNFR-like protein which lacks a transmembrane domain and is secreted as a soluble receptor protein. The protein is most homologous to OPG, which has been identified as a member of the TNFR family which is thought to regulate bone metabolism. The mRNA expression pattern of M68 is examined in multiple human normal and disease tissues. An elevated expression of its mRNA is observed in one third of colon, esophagus, rectum and stomach tumors examined suggesting a role for M68 in cancer.

The present invention also relates to biologically active fragments and/or mutants of M68, comprising the amino acid sequence as set forth in SEQ ID NO:3

and SEQ ID NO:4, including but not necessarily limited to amino acid substitutions, deletions, additions, amino terminal truncations and carboxy-terminal truncations such that these mutations provide for proteins or protein fragments of diagnostic, therapeutic or prophylactic use and would be useful for screening for selective modulators, including but not limited to agonists and/or antagonists for M68 function.

The present invention also relates to polyclonal and monoclonal antibodies raised in response to either the human form of M68, or a biologically active fragment thereof. The polyclonal and/or monoclonal antibodies of the present invention, especially M68 neutralizing antibodies (antibodies that block M68 function and/or block its binding with ligands), will also be useful as therapeutics to modulate M68 expression and/or activity.

The present invention also relates to isolated nucleic acid molecules which are fusion constructions expressing fusion proteins useful in assays to identify compounds which modulate wild-type vertebrate M68.

Therefore, the present invention relates to methods of expressing the human M68 receptor protein and biological equivalents disclosed herein, assays employing these gene products, recombinant host cells which comprise DNA constructs which express these receptor proteins, and compounds identified through these assays which act as agonists or antagonists of M68 activity.

The present invention also relates to assays to screen or select for various modulators of M68 activity, methods of expressing the M68 protein and biological equivalents disclosed herein, recombinant host cells which comprise DNA constructs which express these receptor proteins, and compounds identified through these assays which act as agonists or antagonists of M68 expression and/or activity.

It is one object of the present invention to provide for M68-based assays to select for modulators of this receptor protein. These assays are preferably assays whereby a DNA molecule encoding M68 is transfected or transformed into a host cell, this recombinant host cell is allowed to grow for a time sufficient to express soluble M68 prior to use in various assays described herein.

Therefore, it is an object of the present invention to use M68, cells transfected with an expression vector which directs the expression of M68 or a biological equivalent to screen for modulators, preferably selective modulators, of M68 activity. Any such compound may be useful in the treatment of various cancers, including but not limited to cancers associated with the colon, esophagus, rectum and stomach, as well as in other diseases which are associated with abnormal levels of apoptosis, including but not limited to systemic lupus erythematosis, Hashimoto's thyroiditis, Graves' disease, idiopathic myxoedema, autoimmune diabetes, thrombotic thrombocytopenic purpura, multiple sclerosis, liver diseases (acute and chronic

hepatitis, cirrhosis), autoimmune gastritis, ulcerative colitis, glomerulonephritis, pulmonary fibrosis, heart failure, atherosclerosis, aplastic anaemia and myelodysplastic syndromes, as well as other diseases disclosed herein.

As used herein, "substantially free from other proteins" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other proteins. Thus, an M68 protein preparation that is substantially free from other proteins will contain, as a percent of its total protein, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-M68 proteins. Whether a given M68 protein preparation is substantially free from other proteins can be determined by such conventional techniques of assessing protein purity as, *e.g.*, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with appropriate detection methods, *e.g.*, silver staining or immunoblotting.

As used herein, "substantially free from other nucleic acids" means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other nucleic acids. Thus, a M68 DNA preparation that is substantially free from other nucleic acids will contain, as a percent of its total nucleic acid, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of non-M68 nucleic acids. Whether a given M68 DNA preparation is substantially free from other nucleic acids can be determined by such conventional techniques of assessing nucleic acid purity as, *e.g.*, agarose gel electrophoresis combined with appropriate staining methods, *e.g.*, ethidium bromide staining, or by sequencing.

As used interchangeably herein, "functional equivalent" or "biologically active equivalent" means a receptor which does not have exactly the same amino acid sequence as naturally occurring M68, due to alternative splicing, deletions, mutations, substitutions, or additions, but retains substantially the same biological activity as M68. Such functional equivalents will have significant amino acid sequence identity with naturally occurring M68 and genes and cDNA encoding such functional equivalents can be detected by reduced stringency hybridization with a DNA sequence encoding naturally occurring M68. For the purposes of this invention, naturally occurring M68 has the amino acid sequence shown as SEQ ID NO:3 and is encoded by SEQ ID NOs:1, 2 and 5. A nucleic acid encoding a functional equivalent has at least about 50% identity at the nucleotide level to SEQ ID NOs:1, 2 and 5.

A polypeptide has "substantially the same biological activity" as M68 if that polypeptide has a  $K_d$  for a ligand that is no more than 5-fold greater than the  $K_d$  of M68 having SEQ ID NO:2 for the same ligand.

As used herein, "a conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (*e.g.*, arginine for lysine; glutamic acid for aspartic acid).

As used interchangeably herein, "isolated M68 protein" or "purified M68 protein" refers to M68 protein that has been isolated from a natural source. Use of the term "isolated" or "purified" indicates that M68 protein has been removed from its normal cellular environment. Thus, an isolated M68 protein may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that an isolated M68 protein is the only protein present, but instead means that an isolated M68 protein is substantially free of other proteins and non-amino acid material (*e.g.*, nucleic acids, lipids, carbohydrates) naturally associated with the M68 protein *in vivo*. Thus, an M68 protein that is expressed in a prokaryotic or eukaryotic cell which do not naturally (*i.e.*, without human intervention) express it through recombinant means is an "isolated M68 protein."

As used herein, "TNF" refers to -- tumor necrosis factor --.

As used herein, "TNFR" refers to-- tumor necrosis factor receptor --.

As used herein, the term "mammalian host" will refer to any mammal, including a human being.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-B shows the nucleotide sequence which encodes human M68, as set forth in SEQ ID NO:1.

Figure 2A-B shows the translation of the M68 open reading frame. The nucleotide sequence shown is as set forth in SEQ ID NO:1 (coding strand) and SEQ ID NO:11 (template strand). The amino acid sequence shown is as set forth in SEQ ID NO :3.

Figure 3 shows the amino acid sequence of human M68, as set forth in SEQ ID NO:3.

Figure 4 shows the amino acid sequence of the mature form of human M68, as set forth in SEQ ID NO:4.

Figure 5 shows an alignment of a portion of the amino acid sequence of M68 (as contained within SEQ ID NOs:3 and 4) with the amino acid sequence of a portion of osteoprotegrin (SEQ ID NO:10).

Figures 6A-6B show multi-tissue Northern analysis of mRNA of tumor tissue, normal tissue, and cancer cell lines for determination of expression of mRNA which encodes human M68.

Figure 7A - 7J shows immunohistochemical staining of M68 in normal and tumor tissues of the gastrointestinal tract. Note that strong positive staining in malignant epithelial cells (arrows) in tumor samples (A; colon, F; esophagus, G; stomach, and H; rectum). In normal adjacent colon (E), M68 expression was detected in epithelial cells (arrows) lining the lumen, and was generally absent, or only barely visible in the glandular epithelium. A significant loss of tumor epithelial cell staining was observed when M68 antibody was preincubated with the immunizing peptide (B), and no tumor epithelial cell staining was observed in tumor tissues where the antibody was omitted (C) or substituted for with non-immune rabbit serum (D). Immunohistochemical staining of Fas (CD95) (I) and M68 (J) in a colon adenocarcinoma. Note the similar staining pattern and co-expression of CD95 and M68 in the tumor epithelial cells (arrows).

Figure 8A - 8D shows immunohistochemical scoring of M68 overexpression in formalin-fixed, paraffin embedded sections from GI tract cancer tissues. (A). No staining, or minimal staining, in <10% of the tumor cells: score 0; negative for M68 expression. (B). Faint, barely visible staining in >10% of the tumor cells: score 1+; negative for M68 expression. (C). Weak to moderate staining in >10% of the tumor cells: score 2+; positive for M68 expression. (D). Strong staining in >10% of the tumor cells: score 3+; strongly positive for M68 expression.

Figure 9A - 9E show M68 gene amplification and overexpression. (A, B) a representative of a M68 overexpressing gastrointestinal tumor without M68 gene amplification: (A) immunohistochemical staining of M68 protein expression in a colon adenocarcinoma. (B) dot count using FISH to the adjacent section of with M68 BAC DNA. (C, D) a representative of M68 overexpressing gastrointestinal tumor with M68 gene amplification: (C) immunohistochemical staining of M68 protein expression in a colon adenocarcinoma. (D) dot count using FISH to the adjacent section of with M68 BAC DNA. A significant percentage of cells were with 5 or more dots. (E) TaqMan quantitative PCR analysis of M68 genomic DNA from the same tumor as shown in (A-D). Data shown is the average of two independent experiments.

## DETAILED DESCRIPTION OF THE INVENTION

The M68 protein is a member of a family of proteins which have roles in immune responses, cell death, cell proliferation and differentiation, resulting in testing for potential use in the treatment of a variety of disease including cancer,

inflammatory diseases, and osteoporosis, along with other bone disorders. The altered expression pattern in a multitude of tissues, as disclosed herein, suggests that M68 and its ligand may play a role in cancer by binding to its ligand and blocking apoptotic cell death induced by such a ligand. This anti-apoptotic role of M68 suggests that modulators of M68 will be useful in treatment of apoptosis-related diseases disclosed herein.

Therefore, the present invention relates to methods of using DNA molecules which express human M68 which are useful in identifying modulators of M68 or competing ligands or receptors. An antagonist of M68 will be useful to prevent or inhibit tumorigenesis, especially tumorigenesis effected by interaction of molecules involved in apoptosis in such cancers as cancers of the colon, esophagus, rectum and stomach. Therefore, an antagonist of M68 activity (i.e., antagonizing binding of soluble M68 to its ligand and preventing subsequent receptor binding by the ligand) would also be useful in other diseases associated with an increase in cell survival (i.e., a decrease or inhibition of apoptosis). A compound which acts as an agonist of M68 activity will be useful in the treatment of diseases in which an excessive or inappropriate apoptosis was induced by its ligand. These diseases include but are not limited to autoimmune diseases (such as systemic lupus erythaematosus, Hashimoto's thyroiditis, Graves' disease, idiopathic myxoedema, autoimmune diabetes), inflammation or tissue damage, including indications for the central nervous system such as excitotoxic injury, global ischemia and spinal cord transection, multiple sclerosis, Alzheimer's disease, Parkinsons disease as well as ischemic injury associated with myocardial infarction, stroke, repurfusion injury, pulmonary fibrosis, heart failure, atherosclerosis; thrombotic thrombocytopenic purpura, multiple sclerosis, liver diseases (acute and chronic hepatitis, cirrhosis), autoimmune gastritis, ulcerative colitis, glomerulonephritis and aplastic anaemia and myelodysplastic syndromes. (see Maggi et al, 1998, Pharmacological Research 38(1): 1-34, especially Table III). Therefore, a disease or disorder which is affected by a pathological incidence concerning an increase or decrease in apoptotic activity would be a possible candidate for treatment with the appropriate modulator of M68 activity.

Other potential uses of the components disclosed herein include but are not limited stimulation of cellular differentiation, regulation of immune responses, and regulation of inflammation. Also, the nucleic acid molecules disclosed herein may be used in various diagnostic protocols to measure M68 expression and/or mutation in regard to various cancers, including but not limited to colon, esophagus, rectum and stomach cancer. The DNA molecules disclosed herein may also be uses as a gene therapy vehicles, wherein *in vivo* expression will result in increase levels of the M68

protein, which in turn will combat excess cellular apoptosis associated with the maladies mentioned above.

Therefore, the M68 protein is a member of a family of proteins which have roles in immune responses, cell death, cell proliferation and differentiation, resulting in testing for potential use in the treatment of a variety of disease including cancer, inflammatory diseases, and osteoporosis, as well as other bone disorders. The altered expression pattern in a multitude of tissues, as disclosed herein, suggests that M68 and its ligand may be used for cancer treatment.

The present invention relates to the human gene and isolated cDNA which encodes a protein belonging to the TNFR family. The discovery of this gene was achieved through the identification of sequence similarity between an EST in database and osteoprotegerin (OPG) followed by full length retrieving and sequencing. The mRNA expression pattern of M68 is examined in multiple human normal and disease tissues. An elevated expression of its mRNA is observed in one third of colon, esophagus, rectum and stomach tumors examined, suggesting a role for M68 in cancer.

The present invention relates to an isolated nucleic acid molecule (polynucleotide) which encodes human M68. The nucleic acid molecules of the present invention are substantially free from other nucleic acids. For most cloning purposes, DNA is a preferred nucleic acid.

The present invention relates to an isolated nucleic acid molecule (polynucleotide) which encodes mRNA which expresses a novel human M68, this DNA molecule comprising the nucleotide sequence disclosed herein as SEQ ID NO:1, shown herein as follows:

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CTGACCACTG CAAAGCCAGA GGA CTTCCCC CTGCTGCACA GGTTCAGCAT GTTTGTGCGT
CCACACCACA AGCAGCGCTT CTCACAGACG TGCACAGACC TGACCGGCCG GCCCTACCCG
GGCATGGAGC CACCGGGACC CCAGGAGGAG AGGCTTGCCG TGCCCTCCTGT GCTTACCCAC
AGGGTCCCC AACCAGGCCC CTCACGGTCC GAGAAGACCG GGAAGACCCA GAGCAAGATC
TCGTCTTCC TTAGACAGAG GCCAGCAGGG ACTGTGGGG CGGGCGGTGA GGATGCAGGT
CCCAGCCAGT CCTCAGGACC TCCCCACGGG CCTGCAGCAT CTGAGTGGGG CCTCTAGGAT
GTGCCCAGCC TGCCACACCG CCTCCAGGAA GCAGAGCGTC ATGCAGGTCT TCTGGCCGGA
GCCCCACAAG GACCATGAGG GCGCTGGAGG GGCCAGGCC TCGCTGCTG TGCCTGGTGT
TGGCGCTGCC TGCCCTGCTG CCGGTGCCGG CTGTACGCGG AGTGGCAGAA ACACCCACCT
ACCCCTGGCG GGACGCAGAG ACAGGGGAGC GGCTGGTGTG CGCCAGTGC CCCCAGGCA
CCTTTGTGCA GCGGCCGTGC CGCCGAGACA GCCCCACGAC GTGTGGCCCG TGTCCACCGC
GCCACTACAC GCAGTTCTGG AACTACCTGG AGCGCTGCCG CTACTGCAAC GTCCTCTGCG
GGGAGCGTGA GGAGGAGGCA CGGGCTTGCC ACGCCACCCA CAACCGTGCC TGCCGCTGCC
GCACCGGCTT CTTCGCGCAC GCTGGTTTCT GCTTGGAGCA CGCATCGTGT CCACCTGGTG
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CCGGCGTGAT TGCCCCGGGC ACCCCCAGCC AGAACACGCA GTGCCAGCCG TGCCCCCAG  
 GCACCTTCTC AGCCAGCAGC TCCAGCTCAG AGCAGTGCCA GCCCCACCGC AACTGCACGG  
 CCCTGGGCCT GGCCCTCAAT GTGCCAGGCT CTTCTCCCA TGACACCCTG TGCACCAGCT  
 GCACTGGCTT CCCCCTCAGC ACCAGGGTAC CAGGAGCTGA GGAGTGTGAG CGTGCCGTCA  
 TCGACTTTGT GGCTTTCCAG GACATCTCCA TCAAGAGGCT GCAGCGGCTG CTGCAGGCCC  
 TCGAGGCCCC GGAGGGCTGG GGTCCGACAC CAAGGGCGGG CCGCGCGGCC TTGCAGCTGA  
 AGCTGCGTCG GCGGCTCACG GAGCTCCTGG GGGCGCAGGA CGGGGCGCTG CTGGTGCGGC  
 TGCTGCAGGC GCTGCGCGTG GCCAGGATGC CCGGGCTGGA GCGGAGCGTC CGTGAGCGCT  
 TCCTCCCTGT GCACTGATCC TGGCCCCCTC TTATTTATTC TACATCCTTG GCACCCCACT  
 TGCACTGAAA GAGGCTTTTT TTAAATAGA AGAAATGAGG TTTCTTAA (SEQ ID NO:1).

The above-exemplified isolated DNA molecule, shown in Figure 1A-B and set forth as SEQ ID NO:1, contains 1428 nucleotides. This DNA molecule contains an open reading frame from nucleotide 435 to nucleotide 1334, with a "TGA" termination codon from nucleotides 1335-1337 (Figure 2A-B). This open reading frame encodes a human M68 (Figure 3, SEQ ID NO:3 and Figure 4, SEQ ID NO:4), which shares homology to human osteoprotegerin and TNFR2. The M68 protein contains an open reading frame of 300 amino acids in length, as shown in Figure 2 and as set forth in SEQ ID NO:3.

The present invention also relates to an isolated DNA molecule which encodes an alternatively spliced form from SEQ ID NO:2, which is as follows:

GCAGGGTCCT GTGTCCGCGC TGAGCCGCGC TCTCCCTGCT CCAGCAAGGA CCATGAGGGC  
 GCTGGAGGGG CCAGGCCTGT CGCTGCTGTG CCTGGTGTG GCGCTGCCTG CCCTGCTGCC  
 GGTGCCGGCT GTACGCGGAG TGGCAGAAAC ACCCACCTAC CCCTGGCGGG ACGCAGAGAC  
 AGGGGAGCGG CTGGTGTGCG CCCAGTGCCC CCCAGGCACC TTTGTGCAGC GGCCGTGCCG  
 CCGAGACAGC CCCACGACGT GTGGCCCGTG TCCACCGCGC CACTACACGC AGTTCTGGAA  
 CTACCTGGAG CGCTGCCGCT ACTGCAACGT CCTCTGCGGG GAGCGTGAGG AGGAGGCACG  
 GGCTTGCCAC GCCACCCACA ACCGTGCCTG CCGCTGCCGC ACCGGCTTCT TCGCGCACGC  
 TGTTTTCTGC TTGGAGCACG CATCGTGTC ACCTGGTGCC GGCGTGATTG CCCCGGGCAC  
 CCCCAGCCAG AACACGCAGT GCCAGCCGTG CCCCCAGGC ACCTTCTCAG CCAGCAGCTC  
 CAGCTCAGAG CAGTGCCAGC CCCACGCAA CTGCACGGCC CTGGGCCTGG CCCTCAATGT  
 GCCAGGCTCT TCCTCCCATG ACACCCTGTG CACCAGCTGC ACTGGCTTCC CCCTCAGCAC  
 CAGGGTACCA GGAGCTGAGG AGTGTGAGCG TGCCGTCATC GACTTTGTGG CTTTCCAGGA  
 CATCTCCATC AAGAGGCTGC AGCGGCTGCT GCAGGCCCTC GAGGCCCGG AGGGCTGGGG  
 TCCGACACCA AGGGCGGGCC GCGCGGCCTT GCAGCTGAAG CTGCGTCGGC GGCTCACGGA  
 GCTCCTGGGG GCGCAGGACG GGGCGCTGCT GGTGCGGCTG CTGCAGGCGC TGCGCGTGCC  
 CAGGATGCCC GGGCTGGAGC GGAGCGTCCG TGAGCGCTTC CTCCCTGTGC ACTGATCCTG  
 GCCCCCTCTT ATTTATTCTA CATCCTTGGC ACCCACTTG CACTGAAAGA GGCTTTTTTT  
 TAAATAGAAG AAATGAGGTT TCTTAAAAA AAAAAAAAAA AAAAAA (SEQ ID NO:2).

The M68E clone (SEQ ID NO:2) represents a cDNA molecule which encodes an alternatively spliced form of M68 at 5'UTR. The PCR primers for cloning was derived from this clone. The nucleotides in *italics* (1-44) represent nucleotides which are different than the isolated clone as set forth in SEQ ID NO:1. The open reading frame is as disclosed in SEQ ID NOs:3 and 4, with the initiating Met underlined.

The present invention also relates to isolated DNA molecules which comprise a genomic sequence which comprises the gene which expressing an mRNA which is translated into the M68 protein.

The human genomic sequence for M68 was isolated by screening "down to the well" human BAC clones (Genome Systems) using two pairs of primers derived from M68 cDNA sequence, following manufacture suggested protocols. Human bac clones 168j10 and 186g15 were identified. Clone 168j10 was chosen for sequencing and a partial sequence from 168j10 is disclosed herein as SEQ ID NO:5:

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CCCCCACATCACTTTGGTTCTCTGGCGGGTCAGCTTGGCTCAGTGCCTCAAGGTCGGGTGCCCCCTGC  
CACTGGCTGCGCTTGAGGCTGGCCTTTCTCCAGGAATGTGCTGCGGGTGGAACCCAGGTTCTCTTCTCC  
TTGGGGCCTTTTGCCCCAGAAGCCCATAAATTCCTCAGGCCAACCCGAAATTTTCTCCCTGCTTCCTGCT  
GGGAGCCATTCCCCTCTTCTGCCCATCCCTGCCCTTCAGGCCCCCTGGAGTGAGCTCCAGGTGCAGGCA  
CCAGGCACCTGTGTCCCCTTCTGCCAGCCCCCTCGCTGTGGTCGGACTGTCTTCCCTGGACCTGCTCTT  
ACAAGTCACCACCTGCGAGCCTCATGAGCCGCTGGTGTGACTTGGACAGGACCAAGTTGTGGCACTGTC  
ACCGGGGTGTGCTGTGCCCCCTCCCCGACCTCCATCTTGGCTCAGGGCTCCTTGGGACCATCTTCCC  
TGTGCGTCCAGGTGCTTTGGGACCCAGAGTGTGTGGTTGGGGTCTGTGTGTGGTTGTGAGCTGTGTCC  
TCCTCAGGCCACAGCTGCTCCACCCTGTCCCTCCTGTCTGAGAAGAGGCCGGCAGAAGAACC GCAGG  
AGGGAGGAAGAAGATCCGGCTGGTCAGCCACCCGGTGCGTGAGCTGTCCCTGCACCTGTGCCGACCACC  
ATAGACACGCATGGGAACGCAGCCGTGGGTGCCCCCAGCCACGGCTGGTCCCGATGGGACCAGGGAATC  
CACCCCCAGGAGCTGATGTCCAGGGCAGCTGTGATGCTGACGGCCAGGGGCTCAAGTGTGTGGTTTCTT  
CTGCAGGGGGCTCATGAGTCCCAGCTGGAATCAGGCCCCACCTTGGGCAGGTTTGGCATGGGGCCTGC  
AGCACTGGGCTTGGCCCTGGCATTTCCCTCAAGTGTGGATGCACACCTGCCTCATGTGAGGGACACAGC  
CCATTCCCTAGCCTTGGATCAAAGAACGGAGTTATAGCCGGAGCCAGGAAGCCCCCTGCCTGCTGGA  
CCCCAAGTGTGGCGGCCTTTGTCCATGTCCCTTGGCTTCTGGGAAGAACTGGGTGGTGCCAGGCAGGG  
CTGGTGCCATCAGGAAGTGGGTGGCTGCTGAGGGGCTGGGCTGGCGAGGGCTGGGTGGGGAGTGCCT  
GGGCCGCCCTGCCTTGGTTTCCACGTTTCCGTGTTGGTCTGGGGTGTGTAGAGAGATGGGCACTGCTC  
ATCCGGAAGCCCCCTCCTTGTGCGCTGCCATCCTGGGAGCCTCAGCCGCATCCGCTGTGGGGCAGGGGGC  
TTGAGGGAGGAGGAGAGAGACGGGCCATGCAGGACCCCTGGCTTGAGGCAGAGCCAATCTACCTTTGC  
CCATTCACTGCTCTCAGTTCCTTGCAGCCTCTCACTGTGTGACCTCAGACGGGCCCAGCCCCACAGCT  
TTCTTCCCGCAGCCCCCTCCCTATGTCCATCCAGCCAGCCAGTTTCTCAGGCAGCAGCCCCACCTCGGCA  
GTCAGTGTCCCAGGGAACGCTCAATGTTCCAAGGAAGGCTCTGCAGCCCCAGGGACCAGATGATGAGGC  
TGGCCCTGATGGAGCCTCGGGCCTGTGTCTGCAGGAGGAGCCCGTGGCTGGTGCACAGACGGACAGGG  
CCAAGCTCTTCATGGTGGCCGTGAAGCAGGAGTTGAGCCAAGCCAACCTTGCCACCTTCACCCAGGCC
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TGCAGGACTACAAGGGTTCCGATGACTTCGCCGCCCTGGCCGCCTGTCTCGGCCCCCTCTTTGCTGAGG  
ACCCCAAGAAGCACAACTGCTCCAAGGTGCCCTGGCTTGCAGAGGCCACCCACCTGAGGGCAGTGCT  
GCCGCCGCGTGTGGGGTGGGGGCCATCTGGGTCCAAGGTGGTCTCTGTTCTCTAGAGAAAAAGGGGCAG  
ATGGGGACAGACGCCCCCTTCTCTACAGGCTTCTACCAGTTTGTGCGGCCCCACCATAAGCAGCAGTTT  
GAGGAGGTCTGTATCCAGCTGACAGGACGAGGCTGTGGCTATCGGCCTGAGCACAGCATTCCCCGAAGG  
CAGCGGGCACAGCCGGTCTTGACCCCACTGGTAAATGGGGCCCCAGGTGGGACCCCTCAGACTCCTGCG  
TGGAAGGCAGTGTGGGCCAGAGTCCTGGGCTGCTTGGGGTGGGCATCCTCGGGCCCTGCTTGGCCCCGC  
CTCTCTGTTCCCCATATGGGAGTGATGGGGGCTCCACCTCCACCACCAGCACCAGCAGCACCACCTCCA  
CCTTCACCACCACCACCTCCACCACCACCACCTCCACCACCTCCACCTCCACCACCTCCACCACCTCCA  
CCACCTCCACCACCACCACCTCCACCACCACCACCACCACCACCTCCACCACCACCACCACCACCA  
CCACCTCCACCTCCACCACCTCCACCACCTCCACCACCACCACCACCACCACCTCCACCACCACCA  
CCTCCACCACCACCTCCACCACCACCTCCACCTCCACCACCACCACCTGCACCACCACCTCCACCTCCA  
CCACCACCACCACCTCCACCTCCACCAGCAGCAGCATCACTTGTGGGGAGACCCTGTGCAACTCCATG  
CACAGCCCTGTCCCTGCCATAGCCCCGACCCCTAAGCACAGCCCTGTCCAAGTCCACACGTCCTCCCTGC  
CTCCCATGCATGGTCTGGGGGTCAACTGCACACGCCAGGGTCTTAGGGTCTTAGACCCCTGTCTCTCC  
CTGTTTCTGCCTCTGTTTGGGGTGGAGTCCAAGTCTCCAGAGGCGGAAGCATCTGTGTTTCGTGTGTTAA  
TGAACAGCCCCCTACAGAGTTCCCCTAGTTCACCCAGGGGGGAACCTAGCCTGTGGGACGACCCAGAT  
CCCTTCTGGGCTTGGTACTCACTGGGATATCTCATGCCTGCACCCAGCCTACGGCTCTGAGCTCTCTGA  
GTGGGGCTTTGGCCTGCCCGCCACTGTTCCAGCCCCATCCAGCAGGCTGGTGTCTCCTCTGATGCCCC  
CAGCACCCAGGCGTGTACCTGCCTGGGTTTTCCCGCCCTGGTCTGAGGTGGGTGAGGCCTGGCCTCCCT  
AGCCAGCCCTGCCCCCCCCACCCAGGGAACCTTCCAGATGCTCCCGACCAGCTTTGTGGCTCTACATCT  
CTTCATCAGGAAGAACGGCGCCGGATCCCAAGCTGACCGTGTCCACGGCTGCAGCCCAGCAGCTGGACC  
CCCAAGAGCACCTGAACCAGGGCAGGCCCCACCTGTGCGCCAGGCCACCCCCAACAGGTAGCTGACTCC  
TGAACCGTGTGCAGCCTACGACTTGGTGGGTCCCTCAGTGGCTTCACGAGGCTAACTCTTGAGTGTGGC  
CGGGGCTGCCCCCTGTGGGGAGCCATCTCATGGTGGGGACTGCTCCCGGTTCTGCACCCCGCAGTTGTCC  
TGAGCAGCTCTCCAGGAGTTCCTGGAGGAAGGGCGGGCAGGGCGGTGGGACTCTCAGTCTCCACCCCA  
GCGCCACTCTGAGCCATGCTACTCCCACACCAGGAGACCCTGGCAGCCAACCACAGTGGGGGTCTGGAG  
TGCCCAGAGCAGGGAAGCAGGGCCAGCACGCCGTGAGCGCTACCTGGCTGATGCCCCGAGGGCCCTGG  
GGTCCGCGGGCTGTAGCCAACTCTTGGCAGCGCTGACAGCCTATAAGCAAGACGACGACCTCGACAAGG  
TGCTGGCTGTGTTGGCCGCCCTGACCACTGCAAAGCCAGAGGACTTCCCCCTGCTGCACAGCAAGTGGC  
CCTGGCGTGGGGAACAGCCGGTGGGGTGGGGGGCAGGGGACAAAATGGGGGCTGTGCCGGGTCTGATTG  
AAGCTCCCCGCAGGGTTCAGCATGTTTGTGCGTCCACACCACAAGCAGCGCTTCTCAGACAGCTGCACA  
GACCTGACCGGCCGGCCCTACCCGGGCATGGAGCCACCGGGACCCAGGAGGAGAGGCTTGCCGTGCCT  
CCTGTGCTTACCCACAGGGCTCCCCAACCAGGTAGGGCACCTGCCTGGCTGCTCCTGGCAGCGCCCCAA  
CCGCACGCAGCCCTGGGAGTGAGCAGCAAAGCCCCAGGCCCCCTCAGACTCAAGTCTCTGTCTCCAGG  
CCCCTCACGGTCCGAGAAGACCGGGAAGACCCAGAGCAAGATCTCGTCTTCTTAGACAGAGGCCAGC  
AGGGAAGTGTGGGGGCGGGCGGTGAGGATGCAGGTCCAGCCAGTCTCAGGACCTCCCCACGGGCTGC  
AGCATCTGAGTGGGGTGAGCCTCATGGGAGAGACATCGCTGGGCAGCAGGCCACGGGAGCTCCGGGCGG

GGCCCTCTCAGCAGGCTGTGTGTGCCAGGGCTGTGGGGCAGAGGACGTGGTGCCCTTCCAGTGCCCTGC  
CTGTGACTTCCAGCGCTGCCAAGCCTGCTGGCAACGGCACCTTCAGGTTGGTGCCCTGGCCACTACAGTT  
CCTGCTGGGTGTAGCCCCAGGTGATGGGCTGAGGGGAAAGGGCAGGCCCTTGTCCTGGTGGCAACGCC  
TGGCAGACGTGTGCAGTGGGCCGGTTGTCTCACAGGCCTCTAGGATGTGCCCAGCCTGCCACACCGCCT  
CCAGGAAGCAGAGCGTCATGCAGGTCTTCTGGCCAGAGCCCCAGTGAGTGCCACGGAGGCCCCCAGCA  
CACCCAACGTGGCTTGATCACCTGCCTGTCCAGCTCTGGTGGGCCAAGAACCACCCAACAGAATAGGC  
CAGCCCATGCCAGCCGGCTTGGCCCGCTGCAGGCCTCAGGCAGGCGGGGCCATGGTTGGTCCCTGCGG  
TGGGACCGGATCTGGGCCTGCCTCTGAGAAGCCCTGAGCTACCTTGGGGTCTGGGGTGGGTTTCTGGGA  
AAGTGCTTCCCCAGAACTTCCCTGGCTCCTGGCCTGTGAGTGGTGCCACAGGGGCACCCAGCTGAGCC  
CCTCACCGGGAAGGAGGAGACCCCCGTGGGCACGTGTCCACTTTTAATCAGGGGACAGGGCTCTCTAAT  
AAAGCTGCTGGCAGTGCCCAGGACGGTGTCTTCGTGGCCTGGGCTTGGTGGTGGGAGTTGAGGGACAGG  
GAGTTGGCAGAGGCCCCCTCCAGCCTGCCATGTGACACTGTACTTCTCCACGGTGGGCTCAGCCCTGC  
CCTCATCTCACAGCCGACCCAAGCTGCAGTTGGTAGGGGATCCACCGACACACCAGGCTGCCTGGGC  
TGGTCTCTGGGTGGGAGCTGCCCCAGGTGCTGAGGAGGGCAGCTCCC'TGGCTGGTGAGGCCCCCTCCA  
GAACCACCTTGGACTGAGCTCTGGGGAGGGATGGTACCAGGTGGGTGAGGGGGCTGCCTGGGGAGGG  
AGGGGTTCCTATGGGGCGTGGCGAGGCTGGCCCAGCCCTCTCCCCGCCATATATGTAGGGCAGCAGCA  
GGATGGGCTTCTGGACTTGGGCGGCCCCCTCCGCAGGCGGACCGGGGGCAAGGAGGTGGCATGTGGTC  
AGGCACAGCAGGGTCTGTGTCCGCGCTGAGCCGCGCTCTCCCTGCTCCAGCAAGGACCATGAGGGCGC  
TGGAGGGGCCAGGCCTGTCGCTGCTGTGCCTGGTGTGGCGCTGCCTGCCCTGCTGCCGGTGCCGGCTG  
TACGCGGAGTGGCAGAAACACCCACCTACCCCTGGCGGGACGCAGAGACAGGGGAGCGCTGGTGTGTG  
CCAGTGCCCCCAGGCACCTTGTGTGACGGCCGTGCCGCCGAGACAGCCCCACGACGTGTGGCCCGT  
GTCCACCGCGCCACTACACGCAGTTCTGGAATACCTGGAGCGCTGCCGCTACTGCAACGTCTCTGCG  
GGGAGCGTGAGGAGGAGGCACGGGCTTGCCACGCCACCCACAACCGCGCTGCCGCTGCCGCACCGGCT  
TCTTCGCGCACGCTGGTTTCTGCTTGGAGCACGCATCGTGTCCACCTGGTGCCGGCGTGATTGCCCCGG  
GTGAGAGCTGGGCGAGGGGAGGGGCCCCCAGGAGTGGTGGCCGGAGGTGTGGCAGGGGTACAGTTGCTG  
GTCCCAGCCTTGACCCCTGAGCTAGGACACCAGTTCCCTTGACCCTGTTCTTCCCTCCTGGCTGCAGGC  
ACCCCCAGCCAGAACACGCAGTGCCAGCCGTGCCCCCAGGCACCTTCTCAGCCAGCAGTTCCAGCTCA  
GAGCAGTGCCAGCCCCACCGCAACTGCACGGCCCTGGGCCTGGCCCTCAATGTGCCAGGCTCTTCTCTC  
CATGACACGCTGTGCACCAGCTGCACTGGCTTCCCCCTCAGCACCAAGGTACCAGGTGAGCCAGAGGCC  
TGAGGGGGCAGCACACTGCAGGCCAGGCCCACTTGTGCCCTCACTCCTGCCCTGCACGTGCATCTAGC  
CTGAGGCATGCCAGCTGGCTCTGGGAAGGGGCCACAGTGGATTTGAGGGGTACAGGGTCCCTCCACTAG  
ATCCCCACCAAGTCTGCCCTCTCAGGGGTGGCTGAGAATTTGGATCTGAGCCAGGGCACAGCCTCCCCCT  
GGGGAGCTCTGGGAAAGTGGGCAGCAATCTCCTAACTGCCCGAGGGGAAGGTGGCTGGCTCCTCTGACA  
CGGAGAAACCGAGGCCTGATGGTAACCTCTCCTAACTGCCTGAGAGGAAGGTGGCTGCCTCCTCTGACAT  
GGGGAAACCGAGGCCAATGTTAACCACTGTTGAGAAGTCACAGGGGGAAGTGACCCCCCTTAACATCAA  
GTCAGGTCCGGTCCATCTGCAGGTCCCAACTCGCCCCCTCCGATGGCCCAGGAGCCCCAAGCCCTTGCC  
TGGGCCCCCTTGCTCTTGACGCCAAGGTCCGAGTGGCCACTCTGCCCCCTAGGCCTTTGCTCCAGCT  
CTCTGACCGAAGGCTCCTGCCCTTCTCCAGTCCCCATCGTTGCACTGCCCTCTCCAGCACGGCTCACT

GCACAGGGATTCTCTCTCCTGCAAACCCCCGAGTGGGGCCAGAAAGCAGGGTACCTGGCAGCCCC  
 GCCAGTGTGTGTGGGTGAAATGATCGGACCGCTGCCTCCCCACCCCACTGCAGGAGCTGAGGAGTGTGA  
GCGTGCCGTCATCGACTTTGTGGCTTTCAGGACATCTCCATCAAGAGGCTGCAGCGGCTGCTGCAGGC  
CCTCGAGGCCCCGAGGGCTGGGGTCCGACACCAAGGGCGGGCCGCGCGGCCTTGCAGCTGAAGCTGCG  
TGGGCGGCTCACGGAGCTCCTGGGGGCGCAGGACGGGGCGCTGCTGGTGCGGCTGCTGCAGGCGCTGCG  
CGTGCCAGGATGCCCCGGGCTGGAGCGGAGCGTCCGTGAGCGCTTCCTCCCTGTGCACTGATCCTGGCC  
CCCTCTTATTTATCTACATCCTTGGCACCCCACTTGCAGTGAAGAGGCTTTTTTTTAAATAGAAGAA  
ATGAGGTTTCTTAAAGCTTATTTTTATAAGCTTTTTTCATAAAACTGGTTGTAGTTGCACAGCTACTGG  
 GAGGGCAGCCGGGGACACCTGAGCCGCCCGCTGTGCCAGATCCCTCAGGCTGCCTGCCATCAGAACTG  
 CTGCCCCGGGCTTCCCCTACCTCAGACAGACCCTCCCTGGGAGGATCAGTGGGGAGTGCCACCTCTGCC  
 CCCAGTGGCTGTGGCACGTGGCAGGGGGCCCTGAAGCTCAGCGAGGGTCAGGGCCTGGGAG (SEQ  
 ID NO:5).

Three coding exons are underlined and the starting ATG and stop codon are in bold and underlined. There are additional four exons for the cDNA sequence with the 5' UTR, from nucleotide 3747-3786, 3878-4033, 4140-4292 and 4590-4666 of SEQ ID NO:5. The genomic clone of the present invention is useful in, for example, mutation scanning and gene amplification studies. Regarding mutation scanning, PCR primers can be designed based on the genome sequence and used to identify polymorphism in related disease samples such as autoimmune disease. There are three coding exons for M68 separated by two small introns. In gene amplification studies, PCR primers can be designed based on the genome sequence and used to examine gene amplification with DNA derived from human cancer. Alternatively, probes can be made from the genomic sequence for the purpose of carrying out *in situ* hybridization or southern or dot count hybridization. Another preferred aspect for utilization of the coding sequences disclosed herein as SEQ ID NO:5 comprises the use of non-coding regulatory regions of this gene which are involved in regulation of M68 gene expression in various assays to select for modulators of M68 expression and/or activity. Therefore, non-coding regions are useful in assays to identify regulatory element/factors for M68 transcription and thus to further identify reagents that modulate M68 expression. Such modulators will be especially useful to treat diseases where M68 expression is abnormal. This is especially meaningful, and therefore is a core to the present invention, in view of the fact that this inventor shows that M68 is overexpressed in GI cancer independent of gene amplification, as opposed to a mechanism of M68 overexpression dependent upon M68 gene amplification, which in turn would not suggest involvement of *cis*-acting sequences or *trans*-acting factors in the abnormal expression of M68 and concomitant effect in various cancers.

The present invention also relates to biologically active fragments or mutants of SEQ ID NO:1 which encode mRNA expressing M68. Any such biologically active

fragment and/or mutant will encode either a protein or protein fragment which at least substantially mimics the pharmacological properties of human M68 protein, including but not limited to the human M68 receptor protein as set forth in SEQ ID NO:3 and SEQ ID NO:4. Any such polynucleotide includes but is not necessarily limited to nucleotide substitutions, deletions, additions, amino-terminal truncations and carboxy-terminal truncations such that these mutations encode mRNA which express a protein or protein fragment of diagnostic, therapeutic or prophylactic use and would be useful for screening for agonists and/or antagonists for M68 function.

A preferred aspect of this portion of the present invention is disclosed in Figure 1A-B, a cDNA molecule encoding human M68 (SEQ ID NO:1).

Another preferred aspect of this portion of the present invention is disclosed herein and set forth as SEQ ID NO:2, a human cDNA molecule which comprises a different 5'UTR.

Another preferred aspect of this portion of the present invention is disclosed herein and set forth as SEQ ID NO:5, which comprises an isolated genomic clone encoding human M68.

The isolated nucleic acid molecules of the present invention may include a deoxyribonucleic acid molecule (DNA), such as genomic DNA and complementary DNA (cDNA), which may be single (coding or noncoding strand) or double stranded, as well as synthetic DNA, such as a synthesized, single stranded polynucleotide. The isolated nucleic acid molecule of the present invention may also include a ribonucleic acid molecule (RNA).

The degeneracy of the genetic code is such that, for all but two amino acids, more than a single codon encodes a particular amino acid. This allows for the construction of synthetic DNA that encodes the M68 protein where the nucleotide sequence of the synthetic DNA differs significantly from the nucleotide sequence of SEQ ID NOs: 1, 2 and 5, but still encodes the same M68 protein as SEQ ID NOs: 1, 2 and 5. Such synthetic DNAs are intended to be within the scope of the present invention. If it is desired to express such synthetic DNAs in a particular host cell or organism, the codon usage of such synthetic DNAs can be adjusted to reflect the codon usage of that particular host, thus leading to higher levels of expression of M68 protein in the host. In other words, this redundancy in the various codons which code for specific amino acids is within the scope of the present invention. Therefore, this invention is also directed to those DNA sequences which encode RNA comprising alternative codons which code for the eventual translation of the identical amino acid, as shown below:

A=Ala=Alanine: codons GCA, GCC, GCG, GCU

C=Cys=Cysteine: codons UGC, UGU

D=Asp=Aspartic acid: codons GAC, GAU  
E=Glu=Glutamic acid: codons GAA, GAG  
F=Phe=Phenylalanine: codons UUC, UUU  
G=Gly=Glycine: codons GGA, GGC, GGG, GGU  
H=His =Histidine: codons CAC, CAU  
I=Ile =Isoleucine: codons AUA, AUC, AUU  
K=Lys=Lysine: codons AAA, AAG  
L=Leu=Leucine: codons UUA, UUG, CUA, CUC, CUG, CUU  
M=Met=Methionine: codon AUG  
N=Asp=Asparagine: codons AAC, AAU  
P=Pro=Proline: codons CCA, CCC, CCG, CCU  
Q=Gln=Glutamine: codons CAA, CAG  
R=Arg=Arginine: codons AGA, AGG, CGA, CGC, CGG, CGU  
S=Ser=Serine: codons AGC, AGU, UCA, UCC, UCG, UCU  
T=Thr=Threonine: codons ACA, ACC, ACG, ACU  
V=Val=Valine: codons GUA, GUC, GUG, GUU  
W=Trp=Tryptophan: codon UGG  
Y=Tyr=Tyrosine: codons UAC, UAU.

Therefore, the present invention discloses codon redundancy which may result in differing DNA molecules expressing an identical protein. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally occurring peptide. Methods of altering the DNA sequences include but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand.

The present invention also relates to recombinant vectors and recombinant hosts, both prokaryotic and eukaryotic, which contain the substantially purified nucleic acid molecules disclosed throughout this specification. The nucleic acid molecules of the present invention encoding M68, in whole or in part, can be linked with other DNA molecules, i.e, DNA molecules to which the human M68 are not naturally linked, to form "recombinant DNA molecules" containing the receptor. The

novel DNA sequences of the present invention can be inserted into vectors which comprise nucleic acids encoding human M68 or a functional equivalent. These vectors may be comprised of DNA or RNA; for most cloning purposes DNA vectors are preferred. Typical vectors include plasmids, modified viruses, bacteriophage, cosmids, yeast artificial chromosomes, and other forms of episomal or integrated DNA that can encode a M68 receptor protein. It is well within the skilled artisan to determine an appropriate vector for a particular gene transfer or other use.

Included in the present invention are DNA sequences that hybridize to SEQ ID NOs: 1, 2 and 5 under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows: prehybridization of filters containing DNA is carried out for 2 hours to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hours at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10<sup>6</sup> cpm of <sup>32</sup>P-labeled probe. Washing of filters is done at 37°C for 1 hour in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 minutes before autoradiography. Other procedures using conditions of high stringency would include either a hybridization step carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes. Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, e.g., Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

The present invention also relates to isolated nucleic acid molecules which are fusion constructions expressing fusion proteins useful in assays to identify compounds which modulate wild-type vertebrate M68 activity. A preferred aspect of this portion of the invention includes, but is not limited to, glutathione S-transferase (GST)-M68 fusion constructs which include, but are not limited to, a portion of the soluble human M68 protein as an in-frame fusion at the carboxy terminus of the GST gene by methods known to one of ordinary skill in the art. Recombinant GST-M68 fusion proteins may be expressed in various expression systems, including *Spodoptera frugiperda* (Sf21) insect cells (Invitrogen) using a baculovirus expression vector (pAcG2T, Pharmingen). Since M68 is a secreted protein, two preferred modes for conducting assays to screen for modulators of M68 activity and/or expression exist. First, M68 or an M68-compound complex may be purified and prebound to a solid

matrix (e.g., through antiM68 antibodies or a epitope fusion like GST or Fc). Second, the recombinant M68 protein may be expressed as a fusion protein which comprises a transmembrane domain, thus allowing the binding assay to be carried out in intact functional cells transformed or transfected with the M68-containing fusion construct. Alternatively, the binding assay may be run with crude or purified membrane preparations generated from these same cells transfected with the M68-containing fusion gene of interest.

The present invention also relates to a substantially purified form of the human M68 receptor protein, which comprises the amino acid sequence disclosed in Figure 3 and as set forth in SEQ ID NO:3, as well as Figure 4 and SEQ ID NO:4.

The present invention also relates to biologically active fragments and/or mutants of the human M68 receptor protein comprising the amino acid sequence as set forth in SEQ ID NO:3 and SEQ ID NO:4, including but not necessarily limited to amino acid substitutions, deletions, additions, amino terminal truncations and carboxy-terminal truncations such that these mutations provide for proteins or protein fragments of diagnostic, therapeutic or prophylactic use and would be useful for screening for agonists and/or antagonists of M68 function.

An aspect of the present invention is disclosed in Figure 3 and is set forth as SEQ ID NO:3 in three letter code, and as herein set forth as follows in one letter code:

MRALGPGLS LLCLVLALPA LLPVPAVRGV AETPTYWRD AETGERLVCA QCPPGTFVQR  
PCRRDSPTTC GPCPPRHYTQ FWNLYERCRY CNVLCGEREE EARACHATHN RACRCRTGFF  
AHAGFCLEHA SCPPGAGVIA PGTPSQNTQC QPCPPGTFSA SSSSSEQCQP HRNCTALGLA  
LNVPGSSSHD TLCTSCTGFP LSTRVPGAEE CERAVIDFVA FQDISIKRLQ RLLQALEAPE  
GWGPTPRAGR AALQLKLRRR LTELLGAQDG ALLVRLQLAL RVARMPGLER SVRERFLPVH  
(SEQ ID NO:3), which comprises the amino acid sequence of wild type human M68 receptor protein.

An aspect of the present invention is disclosed in Figure 4 and is set forth as SEQ ID NO:4 in three letter code, and as herein set forth as follows in one letter code:

VAETPTYWR DAETGERLVC AQCPPGTFVQ PCRRDSPTT CGPCPPRHYT QFWNLYERCR  
YCNVLCGERE EEARACHATH NRACRCRTGF FAHAGFCLEH ASCPPGAGVI APGTPSQNTQ  
CQPCPPGTFSA ASSSSSEQCQ PHRNCTALGL ALNVPGSSSH DTLCTSCTGF PLSTRVPGAEE  
ECERAVIDFV AFQDISIKRL QRLQALEAP EGWGPTPRAG RAALQLKLRR RLTELLGAQD  
GALLVRLQLA LRVARMPGLE RSVRERFLPV H (SEQ ID NO:4), which comprises the amino acid sequence of the mature form of wild type human M68 receptor protein.

As with many receptor proteins, it is possible to modify many of the amino acids of M68, particularly those which are not found in the ligand binding domain, and still retain substantially the same biological activity as the original receptor. Thus this invention includes modified M68 polypeptides which have amino acid deletions,

additions, or substitutions but that still retain substantially the same biological activity as M68. Accordingly, the present invention includes polypeptides where one amino acid substitution has been made in SEQ ID NOs:3 and 4 wherein the polypeptides still retain substantially the same biological activity as M68. The present invention also includes polypeptides where two or more amino acid substitutions have been made in SEQ ID NOs:3 and 4 wherein the polypeptides still retain substantially the same biological activity as M68. In particular, the present invention includes embodiments where the above-described substitutions are conservative substitutions. In particular, the present invention includes embodiments where the above-described substitutions do not occur in the ligand-binding domain of M68.

When deciding which amino acid residues of M68 may be substituted to produce polypeptides that are functional equivalents of M68, one skilled in the art would be guided by a comparison of the amino acid sequence of M68 with the amino acid sequences of related proteins, e.g., the human osteoprotegrin as shown in Figure 5 of the present specification. Such a comparison would allow one skilled in the art to minimize the number of amino acid substitutions made in regions that are highly conserved between M68 and the related protein. Accordingly, the present invention includes embodiments where the substitutions are conservative and do not occur in positions where M68 and human osteoprotegrin share the same amino acid (again, see Figure 5).

One skilled in the art would also recognize that polypeptides that are functional equivalents of M68 and have changes from the M68 amino acid sequence that are small deletions or insertions of amino acids could also be produced by following the same guidelines (i.e., minimizing the differences in amino acid sequence between M68 and related proteins). Small deletions or insertions are generally in the range of about 1 to 5 amino acids. The effect of such small deletions or insertions on the biological activity of the modified M68 polypeptide can easily be assayed by producing the polypeptide synthetically or by making the required changes in DNA encoding M68 and then expressing the DNA recombinantly and assaying the protein produced by such recombinant expression.

The present invention also includes chimeric M68 proteins. Chimeric M68 proteins consist of a contiguous polypeptide sequence of M68 fused in frame to a polypeptide sequence of a non-M68 protein.

The present invention also includes M68 proteins that are in the form of multimeric structures, e.g., dimers or trimers.

The present invention also relates to polyclonal and monoclonal antibodies raised in response to either the human form of M68, or a biologically active fragment thereof. The polyclonal and/or monoclonal antibodies of the present invention, especially M68 neutralizing antibodies (antibodies that block M68 function and/or block its binding with ligands), will also be useful as therapeutics to modulate M68 expression and/or activity. In addition, the polyclonal and/or monoclonal antibodies of the present invention are useful as (i) diagnostics for qualitative and/or quantitative detection of M68 expression in a variety of immunoassays [e.g., Western blotting; immunohistochemistry (see Example 4) and immunoprecipitation]; and, (ii) as noted above, inhibition of M68 function using M68 neutralizing antibodies for the treatment of diseases associated with M68 overexpression such as GI cancers or inflammatory diseases. Antibodies that antagonize the effect of M68 (for example, inhibition of M68's ability to protect certain cells from apoptotic cell death or inhibition of M68's ability to bind to its ligand) may be administered directly by methods known in the art [e.g., see *Antibodies in Human Diagnosis and Therapy* by Raven Press, New York (1977)]. Monoclonal antibodies are especially preferred for the treatment of tumors associated with an abnormal M68 expression. For example, an M68 monoclonal antibody which slows the progression of a cancer associated with an increase in M68 expression within cancer cells, and in turn may cause tumor shrinkage over time, will be especially useful. A useful paradigm would be the early success of Herceptin®, a recombinant DNA-derived humanized monoclonal antibody that selectively binds to the extracellular domain of the human epidermal growth factor 2 (HER2). The antibody is produced in CHO cells and the final product is available as a lyophilized powder. HER2 has been shown to be overproduced in 25-30% of primary breast cancers. In turn, administration of Herceptin® has been shown to inhibit the proliferation of tumor cells which overexpress HER2. A prospective M68 monoclonal antibody may be "humanized" by methods well known in the art in order to reduce any unwanted immunological effects of human administration of the antibody. Alternatively, the M68-based antibody may be a chimera, most likely a mouse/human or rat/human chimera. In addition, any such therapeutic M68-based antibody may be administered alone or within a regime that includes other cancer therapies, such as known chemotherapeutic agents, which may act in concert to reduce tumor growth associated with increased M68 expression. Another example of the use of monoclonal antibodies to treat various cancers is Rituxin®, a recombinant DNA-based mouse/human chimeric monoclonal antibody which has been shown to be effective in treating patients with low grade B-cell non-Hodgkin's lymphoma (NHL), a cancer of the immune system. Rituximab targets and destroys white blood cells (B-cells) involved in the disease, resulting in significant tumor shrinkage with less severe

side-effects than most cancer treatments. Additional monoclonal antibodies currently under development include (i) an anti-CD-20 monoclonal antibody to treat patients with low-grade lymphomas, (ii) a combination anti-EGFr antibody with doxorubicin in patients with hormone refractory prostate cancer as well as a combination anti-EGFr antibody with cisplatin in patients with head and neck and lung cancer. It may also be possible to administer an M68 anti-idiotypic antibody so as to stimulate a host immune response to tumors overexpressing M68. Therefore, it is evident that M68 antibodies, especially M68 monoclonal antibodies, are potentially useful tools, alone or in combination with other cancer therapies, for direct therapeutic intervention of cancers characterized by an increase in M68 expression.

The present invention especially relates to polyclonal and/or monoclonal antibodies raised against the peptides selected from the group consisting of NH<sub>2</sub>CRMPGLERSVRERFLPVH-COOH (SEQ ID NO:12), NH<sub>2</sub>VAETPTYPWRDAETGERL-COOH (SEQ ID NO:13), NH<sub>2</sub>LEAPEGWGPTPRAGRA-COOH (SEQ ID NO:14), and NH<sub>2</sub>PPRHYTQFWNYLERC-COOH (SEQ ID NO:15). M68 antibodies may be used directly as therapeutic agents for cancer.

Any of a variety of procedures may be used to clone human M68. These methods include, but are not limited to, (1) a RACE PCR cloning technique (Frohman, et al., 1988, *Proc. Natl. Acad. Sci. USA* 85: 8998-9002). 5' and/or 3' RACE may be performed to generate a full-length cDNA sequence. This strategy involves using gene-specific oligonucleotide primers for PCR amplification of human M68 cDNA. These gene-specific primers are designed through identification of an expressed sequence tag (EST) nucleotide sequence which has been identified by searching any number of publicly available nucleic acid and protein databases; (2) direct functional expression of the human M68 cDNA following the construction of a human M68-containing cDNA library in an appropriate expression vector system; (3) screening a human M68-containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a labeled degenerate oligonucleotide probe designed from the amino acid sequence of the human M68 protein; (4) screening a human M68-containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a partial cDNA encoding the human M68 protein. This partial cDNA is obtained by the specific PCR amplification of human M68 DNA fragments through the design of degenerate oligonucleotide primers from the amino acid sequence known for other kinases which are related to the human M68 protein; (5) screening a human M68-containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a partial cDNA or oligonucleotide with homology to a mammalian M68 protein. This strategy may also involve using gene-specific oligonucleotide primers for PCR amplification of human M68 cDNA identified as an EST as

described above; or (6) designing 5' and 3' gene specific oligonucleotides using SEQ ID NOs: 1, 2 and 5 as a template so that either the full-length cDNA may be generated by known RACE techniques, or a portion of the coding region may be generated by these same known RACE techniques to generate and isolate a portion of the coding region to use as a probe to screen one of numerous types of cDNA and/or genomic libraries in order to isolate a full-length version of the nucleotide sequence encoding human M68.

It is readily apparent to those skilled in the art that other types of libraries, as well as libraries constructed from other cell types-or species types, may be useful for isolating a human M68-encoding DNA or a human M68 homologue. Other types of libraries include, but are not limited to, cDNA libraries derived from other rhesus cells.

It is readily apparent to those skilled in the art that suitable cDNA libraries may be prepared from cells or cell lines which have M68 activity. The selection of cells or cell lines for use in preparing a cDNA library to isolate a cDNA encoding human M68 may be done by first measuring cell-associated M68 activity using any known assay available for such a purpose.

Preparation of cDNA libraries can be performed by standard techniques well known in the art. Well known cDNA library construction techniques can be found for example, in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. Complementary DNA libraries may also be obtained from numerous commercial sources, including but not limited to Clontech Laboratories, Inc. and Stratagene.

It is also readily apparent to those skilled in the art that DNA encoding human M68 may also be isolated from a suitable genomic DNA library. Construction of genomic DNA libraries can be performed by standard techniques well known in the art. Well known genomic DNA library construction techniques can be found in Sambrook, et al., *supra*. Genomic clones containing the M68 gene can be obtained from commercially available human PAC or BAC libraries, *e.g.*, from Research Genetics, Huntsville, AL. Alternatively, one may prepare genomic libraries, especially in P1 artificial chromosome vectors, from which genomic clones containing the M68 can be isolated, using probes based upon the M68 nucleotide sequences disclosed herein. Methods of preparing such libraries are known in the art (Ioannou et al., 1994, *Nature Genet.* 6: 84-89).

This invention also includes vectors containing a M68 gene, host cells containing the vectors, and methods of making substantially pure M68 protein comprising the steps of introducing the M68 gene into a host cell, and cultivating the host cell under appropriate conditions such that M68 is produced and secreted. The

M68 so produced may be harvested in conventional ways. Therefore, the present invention also relates to methods of expressing the human M68 protein and biological equivalents disclosed herein, assays employing these gene products, recombinant host cells which comprise DNA constructs which express these receptor proteins, and compounds identified through these assays which act as agonists or antagonists of M68 activity.

The cloned human M68 cDNA obtained through the methods described above may be recombinantly expressed by molecular cloning into an expression vector (such as pcDNA3.neo, pcDNA3.1, pCR2.1, pBlueBacHis2 or pLITMUS28) containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant human M68. Techniques for such manipulations can be found described in Sambrook, et al., *supra*, are well known and easily available to the artisan of ordinary skill in the art. Therefore, another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences encoding the M68. An expression vector containing DNA encoding a human M68-like protein may be used for expression of human M68 in a recombinant host cell. Such recombinant host cells can be cultured under suitable conditions to produce M68 or a biologically equivalent form. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines.

For instance, one insect expression system utilizes *Spodoptera frugiperda* (Sf21) insect cells (Invitrogen) in tandem with a baculovirus expression vector (pAcG2T, Pharmingen). Also, mammalian species which may be suitable and which are commercially available, include but are not limited to, L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), Saos-2 (ATCC HTB-85), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171) and CPAE (ATCC CCL 209).

A variety of mammalian expression vectors may be used to express recombinant human M68 in mammalian cells. Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned DNA and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic DNA in a variety of hosts such as bacteria, blue green algae, plant cells, insect cells and animal cells. Specifically designed vectors allow the shuttling

of DNA between hosts such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency.

Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Commercially available mammalian expression vectors which may be suitable for recombinant human M68 expression, include but are not limited to, pIRES-hyg (Clontech), pIRES-puro (Clontech), pcDNA3.neo (Invitrogen), pcDNA3.1 (Invitrogen), pCI-neo (Promega), pLITMUS28, pLITMUS29, pLITMUS38 and pLITMUS39 (New England Biolabs), pcDNA1, pcDNA1amp (Invitrogen), pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCtag (ATCC 37460), and IZD35 (ATCC 37565).

Also, a variety of bacterial expression vectors may be used to express recombinant human M68 in bacterial cells. Commercially available bacterial expression vectors which may be suitable for recombinant human M68 expression include, but are not limited to pCR2.1 (Invitrogen), pET11a (Novagen), lambda gt11 (Invitrogen), and pKK223-3 (Pharmacia).

In addition, a variety of fungal cell expression vectors may be used to express recombinant human M68 in fungal cells. Commercially available fungal cell expression vectors which may be suitable for recombinant human M68 expression include but are not limited to pYES2 (Invitrogen) and *Pichia* expression vector (Invitrogen).

Also, a variety of insect cell expression vectors may be used to express recombinant receptor in insect cells. Commercially available insect cell expression vectors which may be suitable for recombinant expression of human M68 include but are not limited to pBlueBacIII and pBlueBacHis2 (Invitrogen), and pAcG2T (Pharmingen).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, protoplast fusion, and electroporation. Transformation is meant to encompass a genetic change to the target cell resulting from an incorporation of DNA. Transfection is meant to include any method known in the art for introducing M68

into the test cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, electroporation, as well as infection with, for example, a viral vector such as a recombinant retroviral vector containing the nucleotide sequence which encodes M68, and combinations thereof. The expression vector-containing cells are individually analyzed to determine whether they produce human M68 protein. Identification of human M68 expressing cells may be done by several means, including but not limited to immunological reactivity with anti-human M68 antibodies, labeled ligand binding and the presence of host cell-associated human M68 activity.

Expression of human M68 DNA may also be performed using *in vitro* produced synthetic mRNA. Synthetic mRNA can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being preferred.

To determine the human M68 cDNA sequence(s) that yields optimal levels of human M68, cDNA molecules including but not limited to the following can be constructed: a cDNA fragment containing the full-length open reading frame for human M68 as well as various constructs containing portions of the cDNA encoding only specific domains of the protein or rearranged domains of the protein. All constructs can be designed to contain none, all or portions of the 5' and/or 3' untranslated region of a human M68 cDNA. The expression levels and activity of human M68 can be determined following the introduction, both singly and in combination, of these constructs into appropriate host cells. Following determination of the human M68 cDNA cassette yielding optimal expression in transient assays, this M68 cDNA construct is transferred to a variety of expression vectors (including recombinant viruses), including but not limited to those for mammalian cells, plant cells, insect cells, oocytes, bacteria, and yeast cells.

Following expression of human M68 in a host cell, M68 protein may be recovered to provide M68 protein in active form. Several M68 protein purification procedures are available and suitable for use. Soluble, secreted recombinant M68 protein may be purified by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography. In addition, recombinant M68 protein can be separated from other proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for full-length M68 protein, or polypeptide fragments of M68 protein.

The present invention is also directed to methods for screening for compounds which modulate the expression of DNA or RNA encoding a human M68 protein. Methods for identifying agonists and antagonists of other receptors are known in the art and can be adapted to identify agonists and antagonists of soluble, secreted M68 protein. The assays described herein can be carried out with cells that have been transiently or stably transfected or transformed with an expression vector which directs expression of M68 or an M68 fusion construct. It will be known that various cell lines may be utilized in the assays described herein. A preferred cell line may be a cell line which expresses a receptor (such as Fas) wherein M68 is known to compete for binding to the associated ligand (such as Fas ligand). Alternatively, the cell line may be co-transfected or transformed with an expression vector which expresses the receptor of interest. Since M68 is a secreted protein it is also possible to use secreted M68 in an assay wherein effective amounts of M68 or M68 fusion protein and an endogenous or cloned receptor (such as Fas) is contacted with test compounds to assay for the ability to effect M68 binding to the expressed receptor. The receptor protein may be supplied as purified protein or a biologically active fragment, or as a whole cell, or a purified, partially purified or cell lysate based membrane preparation which contains the respective receptor protein. Additionally, an M68-based fusion construct may be generated whereby the M68 coding region, or a biologically active portion thereof, is fused to a transmembrane domain, allowing for binding assay to be carried out in intact or lysed cells transformed or transfected with the M68-containing, membrane associated, fusion construct.

In other assays, preferred recombinant-based screening protocols may rely in part on M68 fusion constructs which include but are not limited to an epitope fusion such as GST or Fc, such that soluble M68 fusion protein is generated for use in various assays. Therefore, target cells may be transfected with expression vectors which express M68 or an M68 fusion protein and the transfected cells may be allowed to grow for a time sufficient to allow the soluble M68 receptor to be expressed and secreted. The M68 protein may be harvested and either transferred or precipitated and resuspended into an appropriate assay buffer containing a known radioactively labeled ligand of M68 either in the presence or the absence of the substance, and then measuring the binding of the radioactively labeled known ligand to M68.

Therefore, the present invention relates to methods of expressing M68 in recombinant systems and of identifying modulators M68, namely the ability to interact with various ligands, thus affecting the respective ligand/receptor function. The soluble M68 receptor protein of the present invention is suitable for use in an assay procedure for the identification of compounds which modulate its activity (i.e., modulate the ability of M68 to compete with a ligand for binding to that ligands

respective receptor). Modulating receptor activity, as described herein includes the inhibition or activation of the ability of this soluble, secreted receptor protein to compete with a respective receptors for ligand binding) and also includes directly or indirectly affecting the normal regulation of the receptor activity. Compounds which modulate the receptor activity include agonists, antagonists and compounds which directly or indirectly affect regulation of the receptor activity. When screening compounds in order to identify potential pharmaceuticals that specifically interact with a target receptor, it is necessary to ensure that the compounds identified are as specific as possible for the target receptor. To do this, it is necessary to screen the compounds against as wide an array as possible of receptors that are similar to the target receptor. Thus, in order to find compounds that are potential pharmaceuticals that interact with receptor A, it is necessary not only to ensure that the compounds interact with receptor A (the "plus target") and produce the desired pharmacological effect through receptor A, it is also necessary to determine that the compounds do not interact with receptors B, C, D, etc. (the "minus targets"). In general, as part of a screening program, it is important to have as many minus targets as possible (see Hodgson, 1992, *Bio/Technology* 10:973-980, @ 980). Human M68 proteins and the DNA molecules encoding this receptor protein have the additional utility in that they can be used as "minus targets" in screens designed to identify compounds that specifically interact with other related receptors.

The specificity of binding of compounds having affinity for M68 is shown by measuring the affinity of the compounds for recombinant cells expressing the cloned M68 soluble receptor from these cells. Expression of the cloned receptor and screening for compounds that bind to M68 or that inhibit the binding of a known, radiolabeled ligand of M68 to these cells provides an effective method for the rapid selection of compounds with high affinity for M68. Where binding of a test substance such as an agonist or antagonist to M68 activity is measured, such binding can be measured by employing a labeled substance or agonist. The substance or agonist can be labeled in any convenient manner known to the art, e.g., radioactively, fluorescently, enzymatically. However, such ligands need not necessarily be radiolabeled but can also be nonisotopic compounds that can be used to displace bound radiolabeled compounds or that can be used as activators in functional assays. Compounds identified by the above method are likely to be agonists or antagonists of M68 (again, effecting the ability of M68 to compete with a ligand for binding to that ligands respective receptor). Such compound may be a peptide, protein, or non-proteinaceous organic molecule. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding human M68, or by acting as an agonist or antagonist of the M68 soluble receptor protein. These compounds that

modulate the expression of DNA or RNA encoding human M68 or the biological function thereof may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample. Kits containing human M68, antibodies to human M68, or modified human M68 may be prepared by known methods for such uses.

To this end, the present invention relates in part to methods of identifying a substance which modulates M68 receptor activity, which involves:

(a) combining a test substance in the presence and absence of a M68 receptor protein wherein said M68 receptor protein comprises the amino acid sequence as set forth in SEQ ID NO:2 or SEQ ID NO:4;

(b) incubating the test substance and the soluble M68 receptor protein in the presence of a cell line transfected or transformed with a gene which encodes a TNFR-related protein such as Fas, p55R, p75, CD40, or CD30, along with the respective ligand associated with the recombinantly expressed receptor protein; and,

(c) measuring and comparing the effect of the test substance in the presence and absence of the M68 receptor protein on cell survival of the transfected or transformed cell line expressing the TNFR-related receptor protein in the presence of its respective ligand.

It will of course be within the purview of the skilled artisan to alternatively utilize a cell line which endogenously expresses effective amounts of the TNFR-related receptor protein, so as to negate the requirement to transfect or transform a cell line with an expression vector which recombinantly expressed the TNFR-related receptor protein.

It is also within the purview of the skilled artisan to carry out such assays with an M68-compound complex that may be purified and prebound to a solid matrix such as through antiM68 antibodies or a epitope fusion like GST or Fc.

In addition, several specific embodiments are disclosed herein to show the diverse type of screening or selection assay which the skilled artisan may utilize in tandem with an expression vector directing the expression of the M68 receptor protein or M68 fusion protein. Methods for identifying agonists and antagonists of other receptors are known in the art and can be adapted to identify agonists and antagonists of M68. Therefore, these embodiments are presented as examples and not as limitations. To this end, the present invention includes assays by which M68 modulators (such as agonists and antagonists) may be identified. Accordingly, the present invention includes a method for determining whether a substance is a potential agonist or antagonist of M68 that comprises:

- (a) transfecting or transforming cells with an expression vector that directs expression of M68 in the cells, resulting in test cells;
- (b) allowing the test cells to grow for a time sufficient to allow M68 to be expressed and secreted;
- (c) incubating secreted M68 in the presence and in the absence of the substance; and,
- (d) measuring the binding of the labeled ligand to M68; where if the amount of binding of the labeled ligand is less in the presence of the substance than in the absence of the substance, then the substance is a potential modulator of M68. Examples of possible M68 ligands include but are not limited to TNF- $\alpha$ , sTNF- $\alpha$ , OPGL, LT $\alpha$  (TNF- $\beta$ ), TRAIL, FasL, CD40L, CD30L, CD27L, 4-1BBL, and LIGHT. A preferred aspect of this portion of the invention relates to use of an M68 fusion construct, including but by no means limited to such a fusion construct whereby the M68 coding region, or a biologically active portion thereof, is fused to a transmembrane domain, allowing a binding assay to be carried out in intact or lysed test cells of step (a), which will result in test cells which, instead of secreting M68 (or a biologically active portion thereof), retain the M68-fusion protein in the cell membrane. These M68-containing cells may then be incubated in the presence and in the absence of the test substance and, as in step (d) above, measuring the binding of the labeled ligand to M68 may be accomplished. If the amount of binding of the labeled ligand is less or more in the presence of the substance than in the absence of the substance, then the substance is a potential modulator of M68. Examples of possible M68 ligands again include but are not limited to TNF- $\alpha$ , sTNF- $\alpha$ , OPGL, LT $\alpha$  (TNF- $\beta$ ), TRAIL, FasL, CD40L, CD30L, CD27L, 4-1BB, and LIGHT.

The conditions under which step (c) of the method is practiced are conditions that are typically used in the art for the study of protein-ligand interactions: e.g., physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C. The secreted M68 receptor protein may be incubated in the presence of the substance and the labeled ligand. In a modification of the above-described method, step (c) is modified in that the secreted protein is partially purified and resuspended in an appropriate assay buffer and incubated in the presence of test compound.

Most ligands for TNFR are transmembrane proteins, making a known expression cloning protocol useful for ligand identification for TNFRs. This method will be useful for identifying a ligand or ligand(s) of M68. A panel of cell lines can be screened by flow cytometry with M68 protein (or an epitope tagged version of M68, e.g. M68-Fc) and anti M68 or/ anti epitope antibodies. An expression cDNA library can then be made from such cell lines that show binding to M68. An

expression cloning protocol, such as a slide transfection and two-step detection method (Goodwin, R.G. et al., *Cell* 73, 447-456 (1993)) can then be followed to identify cDNA that direct the expression of a cell surface protein that binds to M68 or M68-Fc.

It is also part of the present invention to utilize the non-coding regulatory regions of the human M68 gene (as disclosed in SEQ ID NO:5) to establish an assay to measure the effect of potential modulators on M68 expression. While many variations are known in the art, one such assay, offered as an example and not a limitation, would be a colorimetric assay which utilizes a recombinant host cell transfected with an expression vector comprising a portion of the 5' UTR of M68 fused to the LacZ gene. This expression vector may be transformed or transfected into a cell line such that an assay may be conducted whereby the effect of various test substances are screened for an ability to either up- or down-regulate M68 promoter activity, which is measured as the expression of the LacZ gene and an OD measurement of  $\beta$ -Gal. The following assay may include the following steps:

- (a) transfecting or transforming a host cell with an expression vector which fuses an M68 5' UTR sequence (contained within SEQ ID NO:5) to a reporter gene, resulting in test cells;
- (b) incubating the test cells in the presence of a test substance for a time sufficient for the cells to grow and for the test substance to interact with various intracellular factors;
- (c) measuring the effect of the test substance on M68 gene expression by a colorimetric assay which measures expression off the M68 promoter and comparing expression levels in the absence of addition of such a test substance.

Agonists and antagonists of M68 that are identified by the above-described methods should have utility in the treatment of diseases that involve the inappropriate expression of M68.

Polyclonal or monoclonal antibodies may be raised against human M68 or a synthetic peptide (usually from about 9 to about 25 amino acids in length) from a portion of human M68 as disclosed in SEQ ID NO:3 and 4. Monospecific antibodies to human M68 are purified from mammalian antisera containing antibodies reactive against human M68 or are prepared as monoclonal antibodies reactive with human M68 using the technique of Kohler and Milstein (1975, *Nature* 256: 495-497). Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogenous binding characteristics for human M68. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope, such as those associated with human M68, as described above. Human M68-specific antibodies are raised by immunizing animals

such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with an appropriate concentration of human M68 protein or a synthetic peptide generated from a portion of human M68 with or without an immune adjuvant.

Preimmune serum is collected prior to the first immunization. Each animal receives between about 0.1 mg and about 1000 mg of human M68 protein associated with an acceptable immune adjuvant. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing *Corynebacterium parvum* and tRNA. The initial immunization consists of human M68 protein or peptide fragment thereof in, preferably, Freund's complete adjuvant at multiple sites either subcutaneously (SC), intraperitoneally (IP) or both. Each animal is bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not receive booster injections following the initial immunization. Those animals receiving booster injections are generally given an equal amount of human M68 in Freund's incomplete adjuvant by the same route. Booster injections are given at about three week intervals until maximal titers are obtained. At about 7 days after each booster immunization or about weekly after a single immunization, the animals are bled, the serum collected, and aliquots are stored at about -20°C.

Monoclonal antibodies (mAb) reactive with human M68 are prepared by immunizing inbred mice, preferably Balb/c, with human M68 protein. The mice are immunized by the IP or SC route with about 1 mg to about 100 mg, preferably about 10 mg, of human M68 protein in about 0.5 ml buffer or saline incorporated in an equal volume of an acceptable adjuvant, as discussed above. Freund's complete adjuvant is preferred. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of about 1 to about 100 mg of human M68 in a buffer solution such as phosphate buffered saline by the intravenous (IV) route. Lymphocytes, from antibody positive mice, preferably splenic lymphocytes, are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner, preferably myeloma cells, under conditions which will allow the formation of stable hybridomas. Fusion partners may include, but are not limited to: mouse myelomas P3/NS1/Ag 4-1; MPC-11; S-194 and Sp 2/0, with Sp 2/0 being preferred. The antibody producing cells and myeloma cells are fused in polyethylene glycol, about 1000 mol. wt., at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells on about days

14, 18, and 21 and are screened for antibody production by an immunoassay such as solid phase immunoradioassay (SPIRA) using human M68 as the antigen. The culture fluids are also tested in the Ouchterlony precipitation assay to determine the isotype of the mAb. Hybridoma cells from antibody positive wells are cloned by a technique such as the soft agar technique of MacPherson, 1973, Soft Agar Techniques, in *Tissue Culture Methods and Applications*, Kruse and Paterson, Eds., Academic Press.

Monoclonal antibodies are produced *in vivo* by injection of pristine primed Balb/c mice, approximately 0.5 ml per mouse, with about  $2 \times 10^6$  to about  $6 \times 10^6$  hybridoma cells about 4 days after priming. Ascites fluid is collected at approximately 8-12 days after cell transfer and the monoclonal antibodies are purified by techniques known in the art.

*In vitro* production of anti-human M68 mAb is carried out by growing the hybridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques known in the art.

Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique and radioimmunoassay (RIA) techniques. Similar assays are used to detect the presence of human M68 in body fluids or tissue and cell extracts.

It is readily apparent to those skilled in the art that the above described methods for producing monospecific antibodies may be utilized to produce antibodies specific for human M68 peptide fragments, or full-length human M68.

Human M68 antibody affinity columns are made, for example, by adding the antibodies to Affigel-10 (Biorad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) and the cell culture supernatants or cell extracts containing full-length human M68 or human M68 protein fragments are slowly passed through the column. The column is then washed with phosphate buffered saline until the optical density ( $A_{280}$ ) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6). The purified human M68 protein is then dialyzed against phosphate buffered saline.

The DNA of the present invention, especially the genomic clone as encoding M68 as set forth in SEQ ID NO:5, or hybridization probes based upon the DNA, can

be used in chromosomal mapping studies in order to identify the chromosomal locations of the M68 gene or of genes encoding proteins related to M68. Such mapping studies can be carried out using well-known genetic and/or chromosomal mapping techniques such as, *e.g.*, linkage analysis with respect to known chromosomal markers or *in situ* hybridization. See, *e.g.*, Verma et al., 1988, Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York, NY. After identifying the chromosomal location of the M68 gene or genes encoding proteins related to M68, this information can be compared with the locations of known disease-causing genes contained in genetic map data (such as the data found in the Genome Issue of Science (1994, 265:1981-2144)). In this way, one can correlate the chromosomal location of the M68 gene or of genes encoding proteins related to M68 with the locations of known disease-causing genes and thus help to limit the region of DNA containing such disease-causing genes. This will simplify the process of cloning such disease-causing genes. Also, once linkage between the chromosomal location of the M68 gene or of genes encoding proteins related to M68 and the locations of a known disease-causing gene is established, that linkage can be used diagnostically to identify restriction fragment length polymorphisms (RFLPs) in the vicinity of the M68 gene or of genes encoding proteins related to M68. Such RFLPs will be associated with the disease-causing gene and thus can be used to identify individuals carrying the disease-causing gene. For such chromosomal mapping studies as described herein, it may be advantageous to use, in addition to the DNA of the present invention, the reverse complement of the DNA of the present invention or RNA corresponding to the DNA of the present invention.

Gene therapy may be used to introduce M68 polypeptides into the cells of target organs. Nucleotides encoding M68 polypeptides can be ligated into viral vectors which mediate transfer of the nucleotides by infection of recipient cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, and polio virus based vectors. Alternatively, nucleotides encoding M68 polypeptides can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted transfer using ligand-nucleotide conjugates, lipofection, membrane fusion, or direct microinjection. These procedures and variations thereof are suitable for *ex vivo* as well as *in vivo* gene therapy. Gene therapy with M68 polypeptides will be particularly useful for the treatment of diseases where it is beneficial to elevate M68 activity for the purpose of countering inappropriately high or uncontrolled levels of apoptosis within a specific tissue.

A DNA fragment encoding M68 or mutant versions thereof may be delivered either systemically or to target cells in the proximity of a solid tumor of the mammalian host by viral or non-viral based methods. Viral vector systems which

may be utilized in the present invention include, but are not limited to, (a) adenovirus vectors, including first generation adenovirus vectors and helper dependent adenoviral vector systems; (b) retrovirus vectors; (c) adeno-associated virus vectors; (d) herpes simplex virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; and (i) vaccinia virus vectors. Non-viral methods of delivery include but are not necessarily limited to direct injection of naked DNA, such as a recombinant DNA plasmid expression vector described herein comprising a DNA fragment encoding M68 or mutated forms of M68.

The recombinant adenoviruses are preferably administered by any method known in the art, including but not limited to, direct injection such as intramuscular injection, or intravenous (i.v.). A recombinant adenovirus delivered by i.v. injection will preferentially infect hepatocytes when administered intravenously, where expression persists for approximately 3-4 weeks subsequent to the initial infection. Suitable titers will depend on a number of factors, such as the particular vector chosen, the host, strength of promoter used and the severity of the disease being treated. The skilled artisan may alter the titer of virus administered to the patient, depending upon the method of delivery, disease treated and efficiency of expression from the recombinant virus. A dose in the range of  $10^9$  -  $10^{11}$  pfu adenovirus is expected to be preferred in most instances. The skilled artisan will also realize that the number of viral particles encoding the transgene, whether or not replication competent in a complementing host cell, are a relevant dosing unit. In most Adenovirus constructs, there are 50 to 100-fold more DNA containing particles than pfus.

The present invention also relates to methods of using the M68 protein, or a biologically active fragment, for inhibition of Fas-mediated apoptosis in a mammalian host, especially a human host, wherein M68 competes with Fas for binding to Fas ligand, thereby affecting a decrease in Fas/FasL induced apoptosis.

As described throughout, the assays described above can be carried out with cells that have been transiently or stably transfected or stably transformed with expression vectors which encode M68 or an M68-fusion construction. Transfection is meant to include any method known in the art for introducing M68 into the test cells. For example, transfection includes calcium phosphate or calcium chloride mediated transfection, lipofection, infection with a retroviral construct containing M68, and electroporation. Transformation is meant to encompass a genetic change to the target cell resulting from an incorporation of DNA.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen and measure levels of human M68. To this end, as exemplified in Example Section 4, M68 immunohistochemistry and/or

RNA measurement will be a useful tool for detection of GI-based cancer, since the data of Example Section 4 shows a strong association of M68 overexpression with cancer. Therefore, the recombinant proteins, DNA molecules, RNA molecules and antibodies of the present invention lend themselves to the formulation of kits suitable for the detection and typing of human M68. Such kits are known in the art and would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant M68 or anti-M68 antibodies suitable for detecting human M68 protein or M68 expression. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Additionally, M68 RNA levels can be measured by multiple techniques such as Northern blot analysis (see Example 2); as well as quantitative or semi quantitative RT-PCR as described in many standard PCR protocols. M68 protein levels can be measured by a variety of immunoassays such as Western blotting, ELISA and immunohistological (IHC) assays. The immunohistochemistry protocol used for M68 antibodies on paraffin embedded tissues are described in Example section 4.

The present invention also relates to a non-human transgenic animal which is useful for studying the ability of a variety of compounds to act as modulators of M68. In regard to transgenic animals of this invention, reference is made to transgenes and genes. As used herein, a transgene is a genetic construct including a gene. The transgene is integrated into one or more chromosomes in the cells in an animal by methods known in the art. Once integrated, the transgene is carried in at least one place in the chromosomes of a transgenic animal. Of course, a gene is a nucleotide sequence that encodes a protein, such as one or a combination of the cDNA clones described herein. The gene and/or transgene may also include genetic regulatory elements and/or structural elements known in the art. A type of target cell for transgene introduction is the embryonic stem cell (ES). ES cells can be obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (Evans et al., 1981, *Nature* 292:154-156; Bradley et al., 1984, *Nature* 309:255-258; Gossler et al., 1986, *Proc. Natl. Acad. Sci. USA* 83:9065-9069; and Robertson et al., 1986 *Nature* 322:445-448). Transgenes can be efficiently introduced into the ES cells by a variety of standard techniques such as DNA transfection, microinjection, or by retrovirus-mediated transduction. The resultant transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The introduced ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal (Jaenisch, 1988, *Science* 240: 1468-1474).

A naturally occurring M68 gene is referred to as the native gene, and if it is not mutant, it can also be referred to as wild-type. An altered M68 gene should not

fully encode the same M68 as native to the host animal, and its expression product can be altered to a minor or greater degree, or absent altogether. In cases where it is useful to express a non-native M68 gene in a transgenic animal in the absence of a native M68 gene we prefer that the altered M68 gene induce a null knockout phenotype in the animal. However a more modestly modified M68 gene can also be useful and is within the scope of the present invention. The M68 mutation may be a targeted deletion mutation, a targeted substitution mutation and/or a targeted insertion mutation. However, the preferred mutation is a deletion mutation, and especially preferred is a deletion mutation which results in a deletion of most if not all of the M68 gene. Transgenic animals are generated which have an altered, or preferably, completely deleted M68 gene. M68 gene deletions, gene modifications and or gene insertions can render the native gene nonfunctional, producing a "knockout" transgenic animal, or can lead to a M68 with altered expression or activity. As noted above, a non-human transgenic animal without an activated M68 gene can be used to for testing/screening of modulators of M68 expression and/or activity (modulators such as small molecules or peptides) that may reverse the pathological phenotype which results from the overexpression or deletion of M68.

A preferred deletion mutation may contain a deletion of anywhere from 1 nucleotide to deletion of the entire gene, including the open reading frame and associated *cis*-acting regulatory sequences associated with wild type M68. A smaller deletion within the open reading frame is preferably not divisible by three, so as to result in a frameshift mutation resulting in a protein which most likely is non-functional. It is preferred that any such smaller deletion not divisible by three be targeted toward the 5' region of the open reading frame to increase the possibility of generating a non-functional truncated protein product. However, as noted above, it is preferable that the deletion mutation encompass most if not all of the M68 gene so as to insure prevention of expression of a functional M68 protein.

Therefore, the transgenic animals which are homozygous, heterozygous or hemizygous for a deficient M68 gene are useful for identifying compounds which modulate wild type M68 activity or expression *in vivo* and studying the effects of various compounds which may be imparted through activation or antagonism of M68 as well as controlling the level of expression, whether that level be abnormally low or abnormally high. Therefore, the generation of M68 deficient transgenic non-human animals, including mice, aids in further defining the *in vivo* function(s) of M68. In addition, transgenic animals can be used as a strain for the insertion of human M68 genes and provides an animal model useful in the design and assessment of various approaches to modulating M68 activity and expression. An altered M68 gene should not fully encode the same M68 as native to the host animal, and its expression product can be altered to a

minor or great degree, or absent altogether. However a more modestly modified M68 gene can also be useful and is within the scope of the present invention. The modified cells, embryos and/or non-human transgenic animal of the present invention can also be used as a source of cells for cell culture. These cells can be used for corresponding *in vitro* studies of M68 expression, activity and the modulation thereof. The non-human transgenic animals disclosed herein are useful for drug antagonist or agonist studies, for animal models of human diseases, and for testing of treatment of disorders or diseases associated with M68. Transgenic animals lacking native M68 are useful in characterizing the *in vivo* function(s) of M68. A transgenic animal carrying a non-native M68 in the absence of native M68 is useful for the establishment of a non-human model for diseases involving M68, such as GI-based cancers.

In view of the teachings within this specification, it is within the purview of the artisan of ordinary skill to utilize antisense RNA transgenes, ribozymes or other modulators of RNA expression or other means of modulating M68 RNA production including promoter mutations, and mutations that affect transcription, to partially or totally knock out expression of the mouse M68 protein. The antisense transgene used herein would encode a polynucleotide which is at least partially complementary to all or a part of the host M68 gene and which will hybridize to a target sequence encoded by the host M68 gene, most specifically a mRNA transcript expressed from the host M68 gene. Any such oligonucleotide sequence should be at least about 15 to 30 nucleotides in length and preferably more than about 30 nucleotides, wherein this sequence is substantially complementary to the target host gene. The antisense transgene need not be a total complement, but instead should contain adequate sequence identity such that the expressed antisense RNA transgene will effectively hybridize with the expressed mRNA from the host target gene so as to efficiently inhibit concomitant protein expression. These antisense polynucleotides may be produced by subcloning the sequence of interest into an appropriate gene expression vector and transferring this vector to pluripotent embryonic stem cells which may be used as described herein to generate another form of an M68 deficient non-human transgenic animal.

A type of target cell for transgene introduction is also the embryonic stem cell (ES). ES cells can be obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (Evans et al., 1981, *Nature* 292: 154-156; Bradley et al., 1984, *Nature* 309: 255-258; Gossler et al., 1986, *Proc. Natl. Acad. Sci. USA* 83: 9065-9069; and Robertson et al., 1986, *Nature* 322: 445-448). Transgenes can be efficiently introduced into the ES cells by a variety of standard techniques such as DNA transfection, microinjection, or by retrovirus-mediated transduction. The resultant transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The introduced ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal

(Jaenisch, 1988, *Science* 240: 1468-1474). The use of gene-targeted ES cells in the generation of gene-targeted transgenic mice was described in 1987 (Thomas et al., 1987, *Cell* 51:503-512) and is reviewed elsewhere (Frohman et al., 1989, *Cell* 56:145-147; Capecchi, 1989, *Trends in Genet.* 5:70-76; Baribault et al., 1989, *Mol. Biol. Med.* 6:481-492; Wagner, 1990, *EMBO J.* 9:3025-3032; Bradley et al., 1992, *Bio/Technology* 10:534-539). See also, U.S. Patent No. 5,464,764, issued to Capecchi and Thomas on November 7, 1995, hereby incorporated by reference). Therefore, techniques are available in the art to generate the M68 deficient animal cells, non-human transgenic embryos, non-human transgenic animals and non-human transgenic littermates of the present invention. The methods for evaluating the targeted recombination events as well as the resulting knockout mice are also readily available and known in the art. Such methods include, but are not limited to DNA (Southern) hybridization to detect the targeted allele, polymerase chain reaction (PCR), polyacrylamide gel electrophoresis (PAGE), *in situ* hybridization and Western blots to detect DNA, RNA and protein. Therefore, the M68 deficient animal cells, non-human transgenic embryos, non-human transgenic animals and non-human transgenic littermates of the present invention may be generated by any techniques known in the art, as sampled in the previous paragraph.

Pharmaceutically useful compositions comprising modulators of human M68 may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, modified human M68, or either M68 agonists or antagonists including tyrosine kinase activators or inhibitors.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration.

The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds identified according to this invention as the active ingredient can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal, hepatic and cardiovascular function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

The following examples are provided to illustrate the present invention without, however, limiting the same hereto.

#### EXAMPLE 1

##### Isolation and Characterization of DNA Fragments Encoding M68

EST sequences display sequence similarities to the cysteine repeats of human osteoprotegerin (OPG) (Acc #. U94332), a member of the TNFR-related family, were searched using TBLASTN and TFASTX3. Two EST sequences (GenBank Acc. # AA155701 and AA025672) were identified. The sequence derived from these two ESTs were then used to search for other homologous sequences available in the databases and sequences derived from the same EST clone. An EST cluster (GenBank Acc. #s aa577603, aa603704, aa613366, aa158406, w67560, aa325843, aa155646, aa025673, aa514270, m91489) were identified. The majority of these ESTs represented 3'UTR sequences. cDNA clones thought to contain longer inserts were obtained (GenBank Acc.#s aa577603, aa603704, aa613366, aa158406, w67560) were obtained from IMAGE. Clones were purified and isolated for sequencing (original EST sequence in database were derived from 3' of the cDNA clones). Internal primers were designed based on the first round sequencing information and then used for second round of sequence analysis. This primer-walking procedure resulted in the complete double-strand sequence analysis. Two clones, both derived from human cancer libraries, contain the full open reading frame and were designated M68. cDNA clones from normal human tissues were obtained from a cDNA library made from human lung (Clontech) by PCR using primers derived from the above sequencing(M68.e30.F:

5'-ctctccctgctccagcaaggacat (SEQ ID NO:6) and M68.e956.R:

5'-atcagtgcacagggaggaagcgct (SEQ ID NO:7)). Sequence analysis indicated both clones isolated from the human cancer libraries possess an identical open reading frame to the above two clones derived from tumor tissue. The 5' stop codon indicates that it contains the full open reading frame. Signal peptide analysis suggests that the first 29 amino acid encoded by M68 cDNA consists of a signal peptide which would be cleaved.

Therefore, search of the EST database with the osteoprotegerin (OPG) amino acid sequence identified a group of clones which represented the partial M68 sequence. The full length M68 cDNA was cloned by recovering EST clones and PCR cloning from a normal human lung cDNA library. Two alternate forms of the M68 cDNA were identified in human cancer cDNA libraries (M68E and M68C). The M68 cDNA encodes a protein of 300 amino acids, with a putative signal peptide cleavage site and the four tandem cysteine-rich repeats which are the hallmark of the

TNFR superfamily. Unlike most of the other TNFR family members, however, no transmembrane domain could be identified, suggesting that the M68 protein is secreted.

## EXAMPLE 2

### Northern Analysis of Human M68 Gene Expression

*Primary tumors* - mRNA isolation from surgically removed human tumor samples were provided by BioChain Institute, Inc., San Leandro, CA. Two  $\mu$ g polyA RNA per lane was run on a denaturing formaldehyde 1% agarose gel, and transferred to a charged-modified nylon membrane. For each type of tissue, RNA were isolated from three different donor's tumors and a normal control tissue, as well as to known human cancer cell lines. The probe was made using a M68 cDNA fragment and labeled via the  $^{32}$ P dCTP random priming method (Ambion). Hybridization was carried out by using Clontech's quick hybridization solution and the manufacture suggested protocols except final wash was done at 55°C rather than 50°C. Membranes were exposed to X-ray film with intensify screen at -80°C overnight. T: tumor tissues; N: normal tissues. The same blot was hybridized to a human ubiquitin probe to verify RNA loading in each lane (Figure 6A). Human normal tissue blots were obtained from Clontech. Hybridization and washing conditions follow manufacture suggested protocols. The same blot was hybridized to a human  $\beta$ -actin probe to verify RNA loading in each lane. The results are shown in Figure 6B.

Numerous reports have shown genomic amplifications of 20q13 in breast (Schwendel et al., 1998, *Br J Cancer* 78: 806-811), gastric (Sakakura et al., 1999, *Genes Chromosomes Cancer* 24: 299-305) colon and lung tumors (Muleris et al., 1995, *Genes Chromosomes Cancer* 14: 155-163.), and in neuroblastomas (Altura et al., 1997, *Genes Chromosomes Cancer* 19: 176-184). This chromosomal localization (see Example 3) and the high incidence of cDNA clones derived from cancer libraries prompted examination of M68/DcR3 expression in normal and tumor tissues, and in human cancer cell lines. To assay for M68 mRNA overexpression, Northern blot analysis was carried out on a series of human tumors. Overexpression of M68 mRNA up to 20 fold was observed in tumors from every level of the GI tract, including esophagus (1/3 tumors), stomach (1/3), rectum (2/3), colon(1/3) (Figure 6A). No M68/DcR3 mRNA overexpression was seen in any of three breast, lung or uterus tumor (data not shown). Several human cancer cell lines also showed M68 overexpression, including the human colonic adenocarcinoma cell lines SW480 (Figure 6A) and SW1116, but not in SW403, HT29 and SW948 (data not shown). M68 mRNA was also detected at a low level by Northern blot in normal human stomach, spinal cord, lymph node, trachea, spleen, colon, and lung as a predominant

1.4 kb message and a minor form at 2.4kb (Figure 6B). The predominant 1.4 kb transcript is equal in size to the longest cDNA clone obtained. Comparison of this clone to the genomic DNA sequence indicated that it is not an alternatively spliced form which deleted a transmembrane domain, but rather had properties of a secreted protein.

#### EXAMPLE 3:

##### Characterization and Chromosomal Localization of the Gene Encoding M68

To map the position of M68 in the human genome, the primers C68.36F:  
5' -CACAGGTTTCAGCATGTTTGTGCGTC (SEQ ID NO:8) and C68.275R:  
5' -CACAGTCCCTGCTGGCCTCTGTCTA (SEQ ID NO:9) were used to carry out PCR reactions with the 83 clones of the Stanford radiation hybrid panel (Cox et al., 1990, *Science*, 250:245:250). The PCR results were scored and submitted to the Stanford Genome Center for linkage analysis. The same procedure was also carried out with the 93 clones of the MIT GeneBridge 4 panel (Research Genetics) and results were submitted to MIT for linkage analysis. Both analysis gives consistent linkage position. M68 was placed to 26cR from D20S173 at chromosome 20 that corresponds to the cytogenetic location of 20q13.3, a chromosomal region frequently amplified in breast and colon tumor cells.

Therefore, M68/DcR3 was mapped using the Genebridge4 radiation hybrid panel to the extreme telomere of chromosome 20, at 20q13.3, 28cR from D20S173 with a lod score of 13. To confirm the RH data and identify additional genes in the region, a BAC clone that contained the full-length M68/DcR3 gene was isolated using PCR probes specific for the 5'UTR and 3'UTR sequences of M68/DcR3. One positive BAC clone, hbm168, was identified and used to independently map M68/DcR3 by fluorescence in situ hybridization FISH to the telomeric end of the q arm of chromosome 20, consistent with the radiation hybrid mapping result. A human chromosome 20  $\alpha$ -satellite probe was used to verify the identity of chromosome 20 (data not shown).

#### EXAMPLE 4

##### M68 Expression Characteristics

*Antibody Production* - Four peptides were used for antibody production: (1) *HM68A*, corresponding to the C-terminus of M68, with an additional Cys residue added to the N-terminus for KLH coupling, (NH<sub>2</sub>-CRMPGLERSVRERFLPVH-COOH [SEQ ID NO:12]); (2) *HM68B*, corresponding to the N-terminus of M68, immediately after the signal peptide cleavage site, (NH<sub>2</sub>-VAETPTYPWR

DAETGERL-COOH [SEQ ID NO:13]); (3) *HM68C*, (NH<sub>2</sub>-LEAPEGWGPTPRAGRA-COOH [SEQ ID NO:14]), and (4) *HM68D*, (NH<sub>2</sub>-PPRHYTQFWNYLERC-COOH [SEQ ID NO:15]). The immunohistochemistry data shown in this example were obtained with antibodies generated from the HM68A peptide. Rabbit antisera were generated according to standard protocols. For characterization of the antisera, the M68 ORF was fused to Fc in frame to generate a M68-Fc fusion in pcDNA3.1 (Invitrogen), and stable CHO cell lines that express the M68-Fc fusion protein were generated. Cells were incubated in serum-free (OPTI-MEM) media for three days, after which conditioned media was harvested. The conditioned media were denatured in SDS sample buffer and subjected to SDS/PAGE prior transferred to nitrocellulose membranes for immunoblot analysis. M68 antisera were used at 1:5000 dilution, followed by HRP conjugated anti-rabbit IgG secondary antibody (Amersham, 1:5000) and ECL detection.

Formalin-fixed, paraffin-embedded blocks from 68 cases of human adenocarcinoma, including primary malignant tumors from esophagus, stomach, colon, and rectum, and thirty normal GI tract tissues were obtained from the Cooperative Human Tissue Network (Eastern Division, Philadelphia, PA; Southern Division, Birmingham, AL; Western Division, Cleveland, OH), and the National Disease Research Interchange (Philadelphia, PA). Five micron sections were cut and mounted onto Superfrost Plus™ slides (Fisher Scientific, Pittsburgh, PA). Sections were pretreated by microwave antigen retrieval (Biogenex, San Ramon, CA), incubated in M68 antibody (1:7000), rinsed, and sequentially incubated with biotinylated rabbit IgG (1:200; Boehringer Mannheim, Indianapolis, IN) and HRP-conjugated avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA). Sections were processed using the catalyzed reporter deposition method (Renaissance® Tyramide Signal Amplification, NEN™, Boston, MA) and the end product was detected with DAB/NiCl<sub>2</sub>. The slides were counterstained with nuclear fast red (Vector). The slides were independently reviewed and graded (0 – 3+) according to HER2/*neu* criteria (15) by two investigators. Images were obtained with a SPOT CCD camera mounted on a Nikon E1000 microscope. Immunodetection of Fas (CD95) was accomplished by incubating sections with monoclonal anti-CD95, APO1/Fas antibody (DAKO Corp., Carpinteria, CA) for 1 hr. after proteinase K treatment. The sections were processed using catalyzed reporter deposition and counterstained as described above. The step by step outline for the above protocol is as follows: (i) deparaffinize and hydrate through graded ETOH to dH2O; (ii) block in 3% H2O2/MeOH for 30 min.; (iii) wash well in dH2O; (iv) microwave in Biogenex Glyca AR solution for 3 min @ full power to boiling, followed by 10 min. power 3

(cycled boiling); (v) cool at RT 30 min.; (vi) block with 1X casein (Vector) plus 10% non-immune sheep serum 1 hr. @ RT; (vii) incubate with rabbit anti-M68, 1199 (1:7000) in blocking buffer overnight @ RT; (viii) wash with PBS 3x @ 5 min.; (ix) incubate with anti-rabbit IgG F(ab')<sub>2</sub> fragment (Boehringer Mannheim Cat. No. 1214659) 1:800, for 30 min. @ RT.; (x) wash with PBS 3x for 5 min.; (xi) incubate with ABC/HRP (Vector) for 30 min. @ RT.; (xii) wash with TBST 3x for 5 min.; (xiii) incubate with Biotinyl-Tyramide (NEN) 1:50 for 10 min.; (xiv) wash with TBST 3x for 5 min.; (xv) incubate with ABC/HRP (Vector) for 30 min. @ RT.; (xvi) wash with TBST 3x for 5 min.; (xvii) incubate with DAB/Nickel Chloride – Monitor under microscope for signal development; (xvii) wash in dH<sub>2</sub>O; (xix) counterstain with Nuclear Fast Red (Vector) for 5 min.; and, (xx) wash in running H<sub>2</sub>O. Dehydrate, clear and mount.

*Overexpression of M68 in GI cancers* - As the level of M68 mRNA appears to be elevated in a subset of human GI tract cancers, M68 was measured at the protein level. Synthetic M68 peptides were coupled to KLH or MAP and used to raise antibodies in rabbits. Antisera were characterized by ELISA with antigen peptide, and Western blot analysis of M68 fusion protein. Antisera that recognized only a single band of the predicted molecular mass by Western blot were used to carry out immunohistochemical analyses on a large series of human normal and pathologically characterized tumor tissues. In normal tissues at all levels of the gastrointestinal tract, M68 immunoreactivity was seen in epithelial cells, in a punctuate staining pattern suggestive of endoplasmic reticulum, a common location for immunostaining of secreted proteins. In many tumor samples, both the number of cells and the intensity of M68 immunoreactivity was dramatically increased relative to control tissues, though the signal remained exclusively epithelial (Figure 7A-J). Specificity of the M68 antiserum was demonstrated by (a) the absence of staining using control antibodies, (b) the significant reduction in staining by preincubating antibodies with antigen peptide, and (c) a similar staining pattern being seen with an antiserum generated with a different M68 antigen peptide.

*Coexpression of M68 with Fas* - If M68 plays a role as a “decoy” receptor, binding to FasL and preventing FasL binding to Fas in tumor cells and thereby preventing Fas-induced apoptosis in tumors, then Fas should be expressed in the GI tract epithelium in close proximity to M68. To test this hypothesis, Fas immunohistochemistry was carried out on adjacent sections of the colon tumors in which M68 overexpression was seen. Fas was found to be expressed in colonic epithelium in a pattern similar to that of M68 (Figure 7I, J). While M68 expression was elevated in tumors, Fas expression in tumors was unchanged or diminished compared to control tissues, suggesting that M68 may alter local Fas concentration as

well as function in tumors. If coexpressed with Fas in normal tissue M68 should act as a guard against tissue damage caused by Fas stimulation.

To investigate whether induced overexpression of M68 could be of benefit in a pathological situation, a 1<sup>st</sup> generation adenovirus expressing the M68 fusion protein from the CMV promoter was constructed. The cDNA of M68 fused Fc was cloned between the human CMV promoter and the bovine growth hormone polyadenylation signal of pcDNA3 (Invitrogen, Carlsbad, CA) and cloned into the adenovirus shuttle vector pΔE1sp1A (Bett et al., 1994, *Proc. Natl. Acad. Sci. U S A* 91: 8802-8806) with the transcription unit directed towards the packaging signal. The complete virus genome was created by homologous recombination in *E. coli* BJ 5183 (Chartier et al., 1996, *J. Virol* 70: 4805-4810) between the shuttle vector and plasmid pE1-E3+. The virus DNA was released from the plasmid by restriction digestion, transfected into 293 cells by calcium phosphate coprecipitation and plaques were obtained after 9 days. The resulting Ad 5 based virus carries the M68-Fc fusion gene in E1 and has a full length E3-region viruses were amplified, large scale stocks were grown in NUNC-cell factories (NUNC, Rochester, NY) and purified by double Cs-banding. For expression studies cos cells were infected with 3000 particles/cell. Five hours after infection cells were washed twice. Medium was replaced for Opti-MEM and incubated for 72 h. Supernatants were subjected to SDS/PAGE, analyzed by immunoblot and used in 1:5 dilutions for protection of copper treated HepG2 cells. Direct infection of HepG2 was performed at 100part/cell.

Fas induced apoptosis plays an essential role in development of hepatitis (Kondo et al., 1997, *Nat. Med.* 3: 409-413). Whereas in viral hepatitis Fas ligand is released by cytotoxic T lymphocytes, FasL is induced together with Fas in hepatocytes overloaded with copper in Wilson disease (Strand et al., 1998, *Nat. Med.* 4: 588-593). Hep G2 cells provide an *in vitro* model of the disease. Preliminary results show that HepG2 cells treated with 100μM Copper chloride for 48 h can be in part protected from apoptosis by either infection with the M68-adenovirus (as describe above) or incubation with cell culture supernatant from virus infected cells. The effect was similar to that of the apoptosis inhibitor z-FAD-FMK.

To objectively quantify M68 tumor overexpression, a scoring system was developed based on the validated system used for Her2/neu, a protein which is overexpressed in a subset of human breast and ovarian tumors (Wisecarver, 1999, *Am. J. Clin. Pathol.* 111: 299-301). Slides were scored on a four-point scale, with 0 indicating no staining, 1+ indicating faint/ barely perceptible staining in >10% of tumor cells, 2+ indicating weak to moderate staining in >10% of tumor cells, and 3+ indicating strong staining in >10% of tumor cells. Tumors which scored 0 or 1+ were judged as negative, while tumors scored 2+ or 3+ were judged as positive.

Figure 8A-D shows immunohistochemical scoring of M68 overexpression in formalin-fixed, paraffin embedded sections from GI tract cancer tissues. (A). No staining, or minimal staining, in <10% of the tumor cells: score 0; negative for M68 expression. (B). Faint, barely visible staining in >10% of the tumor cells: score 1+; negative for M68 expression. (C). Weak to moderate staining in >10% of the tumor cells: score 2+; positive for M68 expression. (D). Strong staining in >10% of the tumor cells: score 3+; strongly positive for M68 expression. Overall, 44% (30 of 68) of tumors scored 2+ or 3+ on this scale, and were therefore designated as overexpressing M68/DcR3. Analysis by tumor site showed that 5 of 8 primary esophageal tumors, 8 of 28 primary gastric tumors, 17 of 25 primary colon tumors, and 2 of 6 primary rectal tumors showed M68/DcR3 overexpression. Analysis of 10 normal tissues adjacent to these tumors showed absent or very limited expression, indicating that M68 protein overexpression is specific to malignant tissues, and not a result of inter-individual differences.

*M68 Overexpression and M68 Gene Amplification* - Pitti et al. (1998, *Nature* 396: 699-703) reported that M68/DcR3 gene was amplified up to 18 fold in 50% of the 18 lung and 17 colon tumors examined. However, protein levels were not directly assayed, so direct correlation between DcR3 gene amplification and gene expression could not be made. To determine whether M68/DcR3 overexpression was always the consequence of gene amplification, quantitative genomic PCR analysis and dot-counting by FISH (Press et al., 1997, *J Clin Oncol.* 15, 2894-904) on tumors which had shown overexpression of M68 by immunohistochemistry (scored 3+) (Figure 9A-9E).

*Quantitative Genomic PCR* - Surgical specimens were provided as frozen tissues. Genomic DNA was isolated using Qiagen's QiaAmp tissue DNA extraction kit. Quantitative PCR was carried out with M68 specific probes and primers, and normalized to beta-globin gene using a TaqMan (ABI 7700) instrument. Primers for M68 were designed using intron sequence to avoid amplification from M68 mRNA. The M68 specific primers were M68Forward: 5'CCAGCACGGCTCACTGC3' (SEQ ID NO:16); M68Reverse: 5'TTTCTGGGCCCCACTCG3' (SEQ ID NO:17); and the fluorogenic probe was 5'CAGGGATTTCTCTCTCCTGCAAACCCC3' (SEQ ID NO:18);. The beta globin primers were Fbglobin62402: 5'ACCCTTAGGCTGCTGGTGG3' (SEQ ID NO:19), Rbglobin62402: 5'GGAGTGGACAGATCCCCAAA3' (SEQ ID NO:20); and the fluorogenic probe was 5'CTACCCTTGGACCCAGAGGTTCTTTGAGTC3' (SEQ ID NO:21);. For each run, a series of dilutions of genomic DNA pool isolated from peripheral blood leukocytes (PBLs) from multiple normal individuals were used for standardization. A second set of M68 and beta-globin primers and probes gave very similar results.

*Probes* - The M68 Bac (Bacterial Artificial Chromosome) clone, was directly labeled with SpectrumGreen dUTP using a Nick Translation kit from Vysis. The probe was nick translated at 15° C for 16 hr at which point the reaction was stopped by heating for 10 min at 70° C. Between 1 and 5 ng of SpectrumGreen labeled M68 probe was mixed with 25 µg of Human Cot I DNA (Gibco BRL, Gaithersburg, MD) and precipitated with 3M sodium acetate and absolute ethanol. The mixture was frozen at -70°C for one hour and then microcentrifuged at 10,000 G for 15 minutes. The ethanol was discarded and the precipitated DNA pellets were air dried for several hours. 3 µl of water and 7 µl of LSI/WCP Hybridization Buffer (Vysis) were mixed and added to the pellet to make a working probe mixture and placed into a 37 °C water bath overnight. An indirect probe labeling method using digoxigenin and a fluorescein labeled anti-digoxigenin antibody caused high background, thus was not used in this study.

*FISH On Paraffin Embedded Sections* - Paraffin embedded sections (4 µ thick) had been fixed in formalin and placed on Probeon Plus slides (Fisher Scientific, Pittsburgh, PA). Prior to hybridization the slides were washed in xylene 3 times for 15 min at room temperature to remove paraffin, followed by washing in absolute ethanol 3 times for 10 min at room temperature. Slides were air dried, then immersed in a pretreatment solution (Vysis) for 30 min at 45°C. Slides were rinsed in room temperature 2XSSC, then treated with Proteinase K (0.25 mg/ml) for 25 to 90 min at 45°. This was followed by 3 washes in 2XSSC at 1 min each. Slides were dehydrated in ethanol, air dried, and placed on a 37°C slide warmer. The probe mixture (10 µl, see Probes section, above) was added to the slide which was coverslipped and sealed. Preparations were denatured in a 90°C oven for 12 min. Slides were hybridized overnight at 37°C in a humidity chamber. The following day the slides were washed with 3 rinses of 50% formamide/2XSSC (pH 7.0) for 10 min each, 1 rinse of 2XSSC for 10 min, then 2 rinses of 2XSSC/0.1%NP-40 for 10 min each. All rinses were at 45 °C. Slides were removed from 2XSSC/0.1%NP-40 and counterstained with 18 µl of 0.6 mg/ml propidium iodide/antifade solution (Vysis), coverslipped, and blotted. Slides were viewed under an Olympus fluorescent microscope with either a dual band FITC/PI or narrow blue filter. Five counts of 100 non-overlapping nuclei were used to obtain a percentage of cells with no amplification (4 or fewer dots) and a percentage of cells with amplification (5 or more dots).

Quantitative PCR was carried out on genomic DNA from tumors and their matching normal control , with relative M68 gene copy number determined normalization to the β-globin gene. Each round of PCR was run with a series of dilutions of standard DNA as control. Unexpectedly, only one out of 6 tumors show

above 2 fold amplification. Three out of 6 tumors show little amplification and the rest two tumors show minimal amplification (Figure 9E). Failure to detect significant M68 gene amplification in these tumor samples is not due to the possible PCR inhibitory factors present in our tumor DNA prep. When M68 BAC DNAs at comparable mole ratio to genomic DNA were added to the tumor DNA samples, we detected M68 gene amplification at the expected level (data not shown). To confirm these results, and address the possibility that the apparent lack of amplification was due to the presence of noncancerous cells in the tumor samples that may reduce the sensitivity of PCR based assay, we carried out FISH analysis using sections adjacent to those used for immunohistochemistry which show score3+. Five counts of 100 non-overlapping nuclei were used to obtain a percentage of cells with or without amplification. Three out of 6 tumors showed only the normal two, and occasionally four, dots, indicating normal M68 gene number and consistent with the PCR results (Figure 9A and 9B). The sixth tumor did show M68 gene amplification by FISH, with approximately half of the cells showing more than 5 dots (Figure 9C and 9D). Two tumors show marginal amplification with 20 or 11 out of 500 cells with 5 or more dots. Together, these data suggest that while M68 gene amplification may occur in tumors, it is not necessary for M68 overexpression. Furthermore, the levels of gene amplification measured unlikely accounts for the dramatic increase at mRNA and protein levels of M68 in tumors.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

## WHAT IS CLAIMED:

1. A method of determining whether a substance is a potential modulator of M68, comprising:
  - (a) combining a test substance in the presence and absence of a M68 soluble receptor protein wherein said protein comprises the amino acid sequence as set forth in SEQ ID NO:3 or SEQ ID NO:4; and,
  - (b) measuring and comparing the effect of the test substance in the presence and absence of the M68 soluble receptor protein.
2. The method of claim 1 wherein the amino acid sequence of the M68 receptor protein is contained as a portion of a fusion protein.
3. A method for determining whether a substance is a potential modulator of M68 comprising:
  - (a) transfecting or transforming cells with an expression vector expressing a M68 protein as set forth in SEQ ID NO:3 or SEQ ID NO:4, resulting in test cells;
  - (b) allowing the test cells to grow for a time sufficient to allow M68 to be expressed and secreted;
  - (c) incubating M68 with a labeled ligand of M68 in the presence and in the absence of the substance;
  - (d) measuring the binding of the labeled ligand to M68; where if the amount of binding of the labeled ligand is less or more in the presence of the substance than in the absence of the substance, then the substance is a potential modulator of M68.
4. The method of claim 3 wherein the amino acid sequence of the M68 receptor protein is contained as a portion of a fusion protein.
5. The method of claim 4 wherein the M68 labeled ligand is selected from the group consisting of TNF- $\alpha$ , sTNF- $\alpha$ , OPGL, LT $\alpha$  (TNF- $\beta$ ), TRAIL, FasL, CD40L, CD30L, CD27L, 1BBL and LIGHT.

6. A method for determining whether a substance is a potential modulator of M68 comprising:

- (a) transfecting or transforming cells with an expression vector expressing a M68 fusion protein which comprises at least a portion of the nucleotide sequence as set forth in SEQ ID NO:3 or SEQ ID NO:4 fused to a nucleotide sequence encoding a transmembrane spanning domain such that expression of said expression vector results in anchoring of the M68 fusion protein to the cell membrane, resulting in M68-containing test cells;
- (b) allowing the test cells to grow for a time sufficient to allow M68 to be expressed;
- (c) incubating the test cells with a labeled ligand of M68 in the presence and in the absence of the substance;
- (d) measuring the binding of the labeled ligand to M68.

7. The method of claim 6 wherein the M68 labeled ligand is selected from the group consisting of TNF- $\alpha$ , sTNF- $\alpha$ , OPGL, LT $\alpha$  (TNF- $\beta$ ), TRAIL, FasL, CD40L, CD30L, CD27L, 4-1BBL, and LIGHT.

8. A method for determining whether a substance is capable of modulating M68 gene expression which comprises:

- (a) transfecting or transforming a host cell with an expression vector which fuses an M68 5' UTR sequence (contained within SEQ ID NO:5) to a reporter gene, resulting in test cells;
- (b) incubating the test cells in the presence of a test substance for a time sufficient for the cells to grow and for the test substance to interact with various intracellular factors; and,
- (c) measuring the effect of the test substance on M68 gene expression by a colorimetric assay which measures expression off the M68 promoter and comparing expression levels in the absence of addition of such a test substance.

9. A method of inhibiting Fas-mediated apoptosis in a mammalian host which comprises delivering a DNA vector to said mammalian host, said DNA vector expressing a soluble form of M68 which competes with Fas for binding to Fas ligand, thereby affecting a decrease in Fas/FasL induced apoptosis.

10. The method of claim 9 wherein said DNA vector comprises the nucleotide sequence as set forth in SEQ ID NO:1.

11. The method of claim 10 wherein said mammalian host is a human.
12. The method of claim 11 wherein said DNA vector is a recombinant adenovirus.
13. The method of claim 11 wherein said DNA vector is a recombinant DNA plasmid vector.
14. A method of inhibiting Fas-mediated apoptosis in a mammalian host which comprises delivering a M68 protein, or biologically active fragment thereof, to said mammalian host, wherein the M68 protein competes with Fas for binding to Fas ligand, thereby affecting a decrease in Fas/FasL induced apoptosis.
15. The method of claim 14 wherein said DNA vector comprises the nucleotide sequence as set forth in SEQ ID NO:3.
16. The method of claim 14 wherein said DNA vector comprises the nucleotide sequence as set forth in SEQ ID NO:4.
17. The method of claim 15 wherein said mammalian host is a human.
18. The method of claim 16 wherein said mammalian host is a human.
19. An isolated nucleic acid molecule which comprises the nucleotide sequence as set forth in SEQ ID NO:5.
20. An isolated nucleic acid molecule of claim 19 which consists of the nucleotide sequence as set forth in SEQ ID NO:5.
21. An isolated nucleic acid molecule which comprises the nucleotide sequence as set forth in SEQ ID NO:1 or SEQ ID NO:2.
22. An isolated nucleic acid molecule of claim 21 which consists of the nucleotide sequence as set forth in SEQ ID NO:1 or SEQ ID NO:2.

23. An isolated nucleic acid molecule which comprises the 5' non-coding region of the human M68 gene, disclosed from nucleotide 1 to nucleotide 5582 within SEQ ID NO:5, as follows:

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CCCCCACATCACTTTGGTTCTCTGGCGGGTCAGCTTGGCTCAGTGCCTCAAGGTCGGGTGCCCCTGCT
CACTGGCTGCGCTTGAGGCTGGCCTTTCTCCAGGAATGTGCTGCGGGTGGAACCCAGGTTCTTCTTCC
TTGGGGCCTTTTGGCCCCAGAAGCCCATAAATTCCTCAGGCCAACCCGAAATTTTCTCCCTGCTTCCTGCT
GGGAGCCATTCCCCCTCTTCTGCCCATCCCTGCCCTTCAGGCCCCCTGGAGTGAGCTCCAGGTGCAGGCA
CCAGGCACCTGTGTCCCCCTTCTGCCAGCCCCCTCGCTGTGGTGGGACTGTCTTCCCTGGACCTGCTCTT
ACAAGTCACCACCTGCGAGCCTCATGAGCCGCTGGTGTGACTTGGACAGGACCAAGTTGTGGCACTGTC
ACCGGGGTGTGCTGTGCCCCCTCCCCCGACCTCCATCTTGGCTCAGGGCTCCTTGGGACCATCTTCCC
TGTGCGTCCAGGTGCTTTGGGACCCCAGAGTGTGTGGTTGGGGTCTGTGTGTGGTTGTGAGCTGTGTCC
TCCTCAGGCCCCACAGCTGCTCCACCCTGTCCCTCCTGTCTGAGAAGAGGCCGGCAGAAGAACCGCGAGG
AGGGAGGAAGAAGATCCGGCTGGTCAGCCACCCGGTGCCTGAGCTGTCCCTGCACCTGTGCCGACCACC
ATAGACACGCATGGGAACGCAGCCGTGGGTGCCCCAGCCACGGCTGGTCCCGATGGGACCAGGGAATC
CACCCCCAGGAGCTGATGTCCAGGGCAGCTGTGATGCTGACGGCCAGGGGCTCAAGTGTGTGGTTTCTT
CTGCAGGGGGCTCATGAGTCCCAGCTGGAATCAGGCCCCACCCCTTGGGCAGGTTTGGCATGGGGCCTGC
AGCACTGGGCTTGGCCCTGGCATTTCCCTCAAGTGTGGATGCACACCTGCCTCATGTGAGGGACACAGC
CCATTCCTAGCCTTGGATCAAAGAACGGAGTTATAGCCGGAGCCAGGAAGCCCCCTGCCTGCTGGAAAA
CCCCAAGTGTGGCGGCCCTTTGTCCATGTCCCTTGGCTTCTGGAAGAACTGGGTGGTGCCAGGCAGGG
CTGGTGCCATCAGGAAGTGGGTGGCTGCTGAGGGGCCTGGGCTGGCGAGGGCCTGGGTGGGGAGTGCTT
GGGCCGCCCTGCCTTGGT'TTCCACGT'TTCCGTGTGGTCTGGGGTGTGTAGAGAGATGGGCACTGCTC
ATCCGGAAGCCCCCTCCTTGTGCGCTGCCATCCTGGGAGCCTCAGCCGCATCCGCTGTGGGGCAGGGGGC
TTGAGGGAGGAGGAGAGAGACGGGCCATGCAGGACCCCTGGCTTGAGGCAGAGCCAATCTACCCTTTGC
CCATTCACCTGCTCTCAGTTCCTTGCCAGCCTCTCACTGTGTGACCTCAGACGGGCCCAGCCCCACAGCT
TTCTTCCCGCAGCCCCCTCCCTATGTCCATCCAGCCAGCCAGTTTCTCAGGCAGCAGCCCCACCTCGGCA
GTCACTGTCCCAGGGAACGCTCAATGTTCCAAGGAAGGCTCTGCAGCCCCAGGGACCAGATGATGAGGC
TGGCCCTGATGGAGCCTCGGGCCTGTGTCTGTCAGGAGGAGCCGTGGCTGGTGCACAGACGGACAGGG
CCAAGCTCTTCATGGTGGCCGTGAAGCAGGAGTTGAGCCAAGCCAACCTTGGCCACCTTCACCCAGGCCC
TGCAGGACTACAAGGGTTCCGATGACTTCGCCGCCCTGGCCGCCTGTCTCGGCCCCCTCTTTGCTGAGG
ACCCCAAGAAGCACAACCTGCTCCAAGGTGCCCTGGCTTGAGAGGCCACCCACCTGAGGGCAGTGCT
GCCGCCGCGTGTGGGGTGGGGGCCATCTGGGTCCAAGGTGGTCTCTGTTCTCTAGAGAAAAAGGGGCAG
ATGGGGACAGACGCCCCCTTCTCTACAGGCTTCTACCACTTTGTGCGGCCCCACCATAAGCAGCAGTTT
GAGGAGGTCTGTATCCAGCTGACAGGACGAGGCTGTGGCTATCGGCCTGAGCACAGCATTTCCCCGAAGG
CAGCGGGCACAGCCGGTCTTGACCCCACTGGTAAATGGGGCCCCAGGTGGGACCCTCAGACTCCTGCG
TGGAAGGCAGTGTGGGCCAGAGTCTGGGCTGCTTGGGGTGGGCATCCTCGGGCCCTGCTTGGCCCCGC
CTCTCTGTTCCCTATGGGAGTGATGGGGGCCTCCACCTCCACCACCAGCACCAGCAGCACCACCTCCA
CCTTCACCACCACCACCTCCACCACCACCACCTCCACCACCTCCACCTCCACCACCTCCACCACCTCCA
CCACCTCCACCACCACCACCACCTCCACCACCACCACCACCACCTCCACCACCACCACCACCACCA
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CCACCTCCACCTCCACCACCTCCACCACCTCCACCACCACCACCACCACCACCTCCACCACCACCA  
CCTCCACCACCACCTCCACCACCACCTCCACCTCCACCACCACCACCTGCACCACCACCTCCACCTCCA  
CCACCACCACCACCTCCACCTCCACCAGCAGCAGCATCACTTGTGGGGAGACCCTGTGCAACTCCATG  
CACAGCCCTGTCCCTGCCATAGCCCCGACCCCTAAGCACAGCCCTGTCCAAGTGCACACGTCCCTGCG  
CTCCCATGCATGGTCTCTGGGGGTCAACTGCACACGCCAGGGTCTTAGGGTCTTAGACCCCTGTCTCC  
CTGTTTCTGCCTCTGTTTGGGGTGGAGTCCAAGTCTCCAGAGGCGGAAGCATCTGTGTTCTGTGTAA  
TGAACAGCCCCCTACAGAGTTCCCTTAGTTCACCCAGGGGGGAACCTAGCCTGTTGGGACGACCCAGAT  
CCCTTCTGGGCTTGCTACTCACTGGGATATCTCATGCCTGCACCCAGCCTACGGCTCTGAGCTCTGA  
GTGGGGCTTTGGCCTGCCCGCCACTGTTCCAGCCCCCATCCAGCAGGCTGGTGTCTCTCTGATGCCCC  
CAGCACCAGGCGTGTACCTGCCTGGGTTTTCCCGCCCTGGTCTGAGGTGGGTGAGGCCTGGCTCCCT  
AGCCAGCCCTGCCCCCCCCACCCAGGGAACCTTCCAGATGCTCCCGACCAGCTTTGTGGCTCTACATCT  
CTTCATCAGGAAGAACGGCGCCGGATCCCAAGCTGACCGTGTCCACGGCTGCAGCCCAGCAGCTGGACC  
CCCAAGAGCACCTGAACCAGGGCAGGCCCCACCTGTGCGCCAGGCCACCCCCAACAGGTAGCTGACTCC  
TGAACCGTGTGCAGCCTACGACTTGGTGGGTCCCTCAGTGGCTTCACGAGGCTAACTCTTGAGTGTGGC  
CGGGGCTGCCCTGTGGGGAGCCATCTCATGGTGGGGACTGCTCCCGGTTCTGCACCCCGCAGTTGTCC  
TGAGCAGCTCTCCAGGAGTTCTTGAGGAAGGGCGGGCAGGGCGGTGGGACTCTCAGTCTCCACCCCCA  
GCGCCACTCTGAGCCATGCTACTCCACACCAGGAGACCCTGGCAGCCAACCACAGTGGGGGTCTGGAG  
TGCCCAGAGCAGGAAGCAGGGCCAGCACGCCGTGAGCGCCTACCTGGCTGATGCCCGCAGGGCCCTGG  
GGTCCGCGGGCTGTAGCCAACTCTTGGCAGCGCTGACAGCCTATAAGCAAGACGACGACCTCGACAAGG  
TGCTGGCTGTGTTGGCCGCCCTGACCACTGCAAAGCCAGAGGACTTCCCCCTGCTGCACAGCAAGTGGC  
CCTGGCGTGGGGAACAGCCGGTGGGGTGGGGGCGAGGGGACAAAATGGGGGCTGTGCCGGGTCTGATTG  
AAGCTCCCCGCGAGGGTTTCAGCATGTTTGTGCGTCCACACCACAAGCAGCGCTTCTCACAGACGTGCACA  
GACCTGACCGGGCCGCCCTACCCGGGCATGGAGCCACCGGGACCCAGGAGGAGAGGCTTGCCGTGCCCT  
CCTGTGCTTACCCACAGGGCTCCCCAACCAGGTAGGGCACCTGCCTGGCTGCTCCTGGCAGCGCCCCAA  
CCGCACGCAGCCCTGGGAGTGAGCAGCAAAGCCCCAGGCCCCCCCTCAGACTCAAGTCTCTGTCTCCAGG  
CCCCTCACGGTCCGAGAAGACCGGGAAGACCCAGAGCAAGATCTCGTCCCTTCCTTAGACAGAGGCCAGC  
AGGGACTGTGGGGCGGGCGGTGAGGATGCAGGTCCCAGCCAGTCCCTCAGGACCTCCCCACGGGCTGC  
AGCATCTGAGTGGGGTGAGCCTCATGGGAGAGACATCGCTGGGCAGCAGGCCACGGGAGCTCCGGGCGG  
GCCCCCTCTCAGCAGGCTGTGTGTGCCAGGGCTGTGGGGCAGAGGACGTGGTGCCCTTCCAGTGCCCTGC  
CTGTGACTTCCAGCGCTGCCAAGCCTGCTGGCAACGGCACCTTCAGGTGGTGCTGGCCACTACAGTT  
CCTGCTGGGTGTAGCCCCAGGTGATGGGCTGAGGGGGAAGGGCAGGCCCTTGTCCTGGTGGCAACGCC  
TGGCAGACGTGTGCAGTGGGCCGGTTGTCTCACAGCCCTCTAGGATGTGCCAGCCTGCCACACCGCCT  
CCAGGAAGCAGAGCGTCATGCAGGTCTTCTGGCCAGAGCCCCAGTGAGTGCCACGGAGGCCCCCAGCA  
CACCCAACGTGGCTTGATCACCTGCCTGTCCAGCTCTGGTGGGCCAAGAACCACCCAAACAGAATAGGC  
CAGCCCATGCCAGCCGGCTTGCCCCGTGCAGGCCCTCAGGCAGGCGGGGCCATGGTTGGTCCCTGCGG  
TGGGACCGGATCTGGGCTGCCTCTGAGAAGCCCTGAGCTACCTTGGGGTCTGGGGTGGGTTTCTGGGA  
AAGTGCTTCCCCAGAACTTCCCTGGCTCCTGGCTGTGAGTGGTGCCACAGGGGCACCCAGCTGAGCC  
CCTCACCGGGAAGGAGGAGACCCCCGTGGGCACGTGTCCACTTTTAATCAGGGGACAGGGCTCTCTAAT

AAAGCTGCTGGCAGTGCCCAGGACGGTGTCTTCGTGGCCTGGGCTTGGTGGTGGGAGTTGAGGGACAGG  
 GAGTTGGCAGAGGCCCTCCCAGCCTGCCATGTGACACTGTACTTCCTCCACGGTGGGCTCAGCCCTGC  
 CCTCATCCTCACAGCCGAGCCAAGCTGCAGTTGGTAGGGGATCCACCGACACACCAGGCTGCCTGGGC  
 TGGTCTCTGGGTGGGAGCTGCCCCAGGTGCTGAGGAGGGCAGCTCCCTGGCTGGTGAGGCCCCCTCCA  
 GAACCACCCTTGGACTGAGCTCTGGGGAGGGATGGTACCAGGTGGGTGAGGGGGGCTGCCTGGGGAGGG  
 AGGGGTTCCTATGGGGCCTGGCGAGGCTGGCCAGCCCTCTCCCCGCCATATATGTAGGGCAGCAGCA  
 GGATGGGCTTCTGGACTTGGGCGGCCCTCCGCAGGCGGACCGGGGGCAAAGGAGGTGGCATGTCGGTC  
 AGGCACAGCAGGGTCCCTGTCTCCGCGCTGAGCCGCGCTCTCCCTGCTCCAGCAAGGACCATG  
 (contained within SEQ ID NO:5).

24. An isolated nucleic acid molecule which comprises an intronic or 3' UTR from the human M68 gene, selected from the group of sequences consisting of

(a) GTGAGAGCTGGGCGAGGGGAGGGGCCCCAGGAGTGGTGGCCGGAGGTGTGGCAGGGGTCA  
 GGTGCTGGTCCCAGCCTTGCACCCTGAGCTAGGACACCAGTTCCCTGACCCTGTCTCTCCCTCCTGG  
 CTGCAG;

(b) GTGAGCCAGAGGCTGAGGGGGCAGCACACTGCAGGCCAGGCCACTTGTGCCCTCACTC  
 CTGCCCCTGACGTGCATCTAGCCTGAGGCATGCCAGCTGGCTCTGGGAAGGGGCCACAGTGGATTTGA  
 GGGGTGAGGGGTCCCTCCACTAGATCCCCACCAAGTCTGCCCTCTCAGGGGTGGCTGAGAATTTGGATC  
 TGAGCCAGGGCACAGCCTCCCTGGGGAGCTCTGGGAAAGTGGGCAGCAATCTCCTAACTGCCCCAGGG  
 GAAGGTGGCTGGCTCCTCTGACACGGAGAAACCGAGGCCTGATGGTAACTCTCCTAACTGCCTGAGAGG  
 AAGGTGGCTGCCTCCTCTGACATGGGGAAACCGAGGCCCAATGTTAACCACTGTTGAGAAGTCACAGGG  
 GGAAGTGACCCCTTAACATCAAGTCAGGTCCGGTCCATCTGCAGGTCCCAACTCGCCCCCTCCGATGG  
 CCCAGGAGCCCCAAGCCCTTGCCTGGGCCCCCTTGCCTCTTGCAGCCAAGGTCCGAGTGGCCACTCCTG  
 CCCCCTAGGCCTTTGCTCCAGCTCTCTGACCGAAGGCTCCTGCCCCCTTCTCCAGTCCCCATCGTTGCAC  
 TGCCCCCTCCAGCACGGCTCACTGCACAGGGATTTC'TCTCTCTCTGCAAACCCCCCGAGTGGGGCCCAGA  
 AAGCAGGGTACCTGGCAGCCCCCGCCAGTGTGTGTGGGTGAAATGATCGGACCGCTGCCTCCCCACCCC  
 ACTGCAG; and,

(c) GCTTATTTTATAAAGCTTTTTCATAAACTGGTTGTAGTTGCACAGCTACTGGGAGGGC  
 AGCCGGGGACACCTGAGCCGCCCGCTGTGCCAGATCCCTCAGGCTGCCTGCCATCAGAACTGCTGCCC  
 GGGGCTTCCCTACCTCAGACAGACCCTCCCTGGGAGGATCAGTGGGGAGTGCCACCTCTGCCCCAGT  
 GGCTGTGGCACGTGGCAGGGGCCCCCTGAAGCTCAGCGAGGGTCAGGGCCTGGGAG (contained  
 within SEQ ID NO:5).

25. A substantially purified protein comprising the amino acid sequence as set forth in SEQ ID NO:3.

26. A substantially purified protein comprising the amino acid sequence as set forth in SEQ ID NO:4.

27. The purified protein of claim 25 which consists of the amino acid sequence as set forth in SEQ ID NO:3.
28. The purified protein of claim 26 which consists of the amino acid sequence as set forth in SEQ ID NO:4.
29. An antibody that binds specifically to M68 protein wherein the M68 protein comprises the amino acid sequence as set forth in SEQ ID NO:3 or SEQ ID NO:4.
30. The antibody of claim 29 which generated from the peptide selected from the group consisting of NH<sub>2</sub>-CRMPGLERSVRERFLPVH-COOH (SEQ ID NO:12), VAETPTYPWDRDAETGERL (SEQ ID NO:13), LEAPEGWGPTPRAGRA (SEQ ID NO:14), PPRHYTQFWNYLERC (SEQ ID NO:15).

1/14

1 CTGACCACTG CAAAGCCAGA GGA CTGCTGCACA GGTTCAGCAT  
51 GTTTGTGCGT CCACACCACA AGCAGCGCTT CTCACAGACG TGCACAGACC  
101 TGACCGGCCG GCCCTACCCG GGCATGGAGC CACCGGGACC CCAGGAGGAG  
151 AGGCTTGCCG TGCCTCCTGT GCTTACCCAC AGGGCTCCCC AACCAGGCCC  
201 CTCACGGTCC GAGAAGACCG GGAAGACCCA GAGCAAGATC TCGTCCTTCC  
251 TTAGACAGAG GCCAGCAGGG ACTGTGGGGG CGGGCGGTGA GGATGCAGGT  
301 CCCAGCCAGT CCTCAGGACC TCCCCACGGG CCTGCAGCAT CTGAGTGGGG  
351 CCTCTAGGAT GTGCCCAGCC TGCCACACCG CCTCCAGGAA GCAGAGCGTC  
401 ATGCAGGTCT TCTGGCCGGA GCCCCACAAG GACCATGAGG GCGCTGGAGG  
451 GGCCAGGCCT GTCGCTGCTG TGCCTGGTGT TGGCGCTGCC TGCCCTGCTG  
501 CCGGTGCCCG CTGTACGCGG AGTGGCAGAA ACACCCACCT ACCCCTGGCG  
551 GGACGCAGAG ACAGGGGAGC GGCTGGTGTG CGCCCAGTGC CCCCAGGCA  
601 CCTTTGTGCA GCGGCCGTGC CGCCGAGACA GCCCCACGAC GTGTGGCCCCG  
651 TGTCCACCGC GCCACTACAC GCAGTTCTGG AACTACCTGG AGCGCTGCCG  
701 CTA CTGCAAC GTCCTCTGCG GGGAGCGTGA GGAGGAGGCA CGGGCTTGCC  
751 ACCCCACCCA CAACCGTGCC TGCCGCTGCC GCACCGGCTT CTTGCGGCAC  
801 GCTGGTTTCT GCTTGGAGCA CGCATCGTGT CCACCTGGTG CCGGCGTGAT  
851 TGCCCCGGGC ACCCCCAGCC AGAACACGCA GTGCCAGCCG TGCCCCCAG  
901 GCACCTTCTC AGCCAGCAGC TCCAGCTCAG AGCAGTGCCA GCCCCACCGC  
951 AACTGCACGG CCCTGGGCCT GGCCCTCAAT GTGCCAGGCT CTTCTCCCA  
1001 TGACACCCTG TGCACCAGCT GCACTGGCTT CCCCCTCAGC ACCAGGGTAC  
1051 CAGGAGCTGA GGAGTGTGAG CGTGCCGTCA TCGACTTTGT GGCTTTCCAG  
1101 GACATCTCCA TCAAGAGGCT GCAGCGGCTG CTGCAGGCCC TCGAGGCCCC  
1151 GGAGGGCTGG GGTCCGACAC CAAGGGCGGG CCGCGCGGCC TTGCAGCTGA  
1201 AGCTGCGTCG GCGGCTCACG GAGCTCCTGG GGGCGCAGGA CGGGGCGCTG  
1251 CTGGTGCGCC TGCTGCAGGC GCTGCGCGTG GCCAGGATGC CCGGGCTGGA

FIG. 1A

SUBSTITUTE SHEET (RULE 26)

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1301 GCGGAGCGTC CGTGAGCGCT TCCTCCCTGT GCACTGATCC TGGCCCCCTC  
1351 TTATTTATTC TACATCCTTG GCACCCCACT TGCACTGAAA GAGGCTTTTT  
1401 TTAAATAGA AGAAATGAGG TTTCTTAA (SEQ ID NO:1)

**FIG. 1B**

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1 CTGACCACTGCTCAAAGCCAGAGGACTTCCCCCTGCTGCACAGGTTTCAGCATGTTTGTGCGT 60  
-----+-----+-----+-----+-----+  
GACTGGTGACGTTTCCGTCTCCTGAAGGGGGACGACGTGTCCAAGTCGTACAAACACGCA  
61 CCACACCACAAGCAGCGCTTCTCACAGACGTGCACAGACCTGACCGGCCCGCCCTACCCG 120  
-----+-----+-----+-----+-----+  
GGTGTGCTGTTTCGTTCGGAAGAGTGTCTGCACGTGTCTGGACTGGCCGGCCGGGATGGGC  
121 GGCATGGAGCCACCGGGACCCAGGAGGAGGCTTGCCGTGCCCTCCTGTGCTTACCCAC 180  
-----+-----+-----+-----+-----+  
CCGTACCTCGGTGGCCCTGGGGTCTCTCTCCGAACGGCACGGAGGACACGAATGGGTG  
181 AGGGCTCCCCAACAGGCCCTCACGGTCCGAGAAGACCGGAAGACCCAGAGCAAGATC 240  
-----+-----+-----+-----+-----+  
TCCCGAGGGGTGGTCCGGGAGTGCCAGGCTCTTCTGGCCCTTCTGGGTCTGTTCTAG  
241 TCGTCCTTCTTAGACAGAGGCCAGCAGGGACTGTGGGGCGGGCGGTGAGGATGCAGGT 300  
-----+-----+-----+-----+-----+  
AGCAGGAAGGAATCTGTCTCCGGTCGTCCCTGACACCCCCGCCGCCACTCCTACGTCCA  
301 CCCAGCCAGTCTTCAGGACCTCCCCACGGGCCCTGCAGCATCTGAGTGGGGCCTCTAGGAT 360  
-----+-----+-----+-----+-----+  
GGGTCGGTCAGGAGTCTTGAGGGGTGCCCGACGTCTGAGACTACCCCGGAGATCCTA  
361 GTGCCCAGCCTGCCACACCGCCTCCAGGAAGCAGAGCGTCATGCAGGTCTTCTGGCCGGA 420  
-----+-----+-----+-----+-----+  
CACGGGTCCGACGGTGTGGCGGAGGTCTTCGTCTCGCAGTACGTCCAGAAGACCGGCCCT  
421 GCCCCACAAGGACCATGAGGGCGCTGGAGGGGCCAGGCCCTGTGCTGCTGTGCCTGGTGT 480  
-----+-----+-----+-----+-----+  
CGGGGTGTTCTTGTAATCCCGGACCTCCCGGTCGGACAGCAGACAGGACCACA  
M R A L E G P G L S L L C L V L  
481 TGGCGCTGCCCTGCCCTGCTGCCGGTGCCGGCTGTACGGGAGTGGCAGAAACACCCACCT 540  
-----+-----+-----+-----+-----+  
ACCGGACGGACGGGACGACGGCCACGGCCGACATGCGCCTACCGTCTTTGTGGGTGGA  
A L P A L L P V P A V R G V A E T P T Y  
541 ACCCCTGGCGGGACGCAGACAGGGGAGCGCTGGTGTGCGCCAGTGCCCCCAGGCA 600  
-----+-----+-----+-----+-----+  
TGGGACCGCCCTGCGTCTCTGTCCCCTCGCCGACCACACCGGGTCACGGGGGTCCGT  
P W R D A E T G E R L V C A Q C P P G T  
601 CCTTTGTGCAGCGCCGTGCCGCCGAGACAGCCCCACGACGTGTGGCCCGTGTCCACCGC 660  
-----+-----+-----+-----+-----+  
GGAAACAGTCGCCGGCACGGCGGCTCTGTGCGGTGCTGCACACCGGCCACAGTGGCG  
F V Q R P C R R D S P T T C G P C P P R  
661 GCCACTACACGAGTTCTGGAACCTGAGAGCGTGCCGCTACTGCAACGTCTCTGCG 720  
-----+-----+-----+-----+-----+  
CGGTGATGTGCGTCAAGACCTTGATGGACCTCGCGACGGGATGACGTTGCAGGAGACGC  
H Y T Q F W N Y L E R C R Y C N V L C G  
721 GGGAGCGTGAGGAGGAGGCACGGGCTTGCCACGCCACCCACAACCGTGCTGCCGCTGCC 780  
-----+-----+-----+-----+-----+  
CCCTCGCACTCTCTCCGTGCCCGAACGGTGCGGTGGGTGTTGGCAGGACGGCGACGG  
E R E E E A R A C H A T H N R A C R C R

FIG. 2A

**SUBSTITUTE SHEET (RULE 26)**

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781 GCACCGGCTTCTTCGCGCAGCTGGTTTCTGCTTGGAGCAGCATCGTGTCCACCTGGTG 840  
 -----+-----+-----+-----+-----+-----+  
 CGTGGCCGAAGAAGCGCTGCGACCAAGACGAACCTCGTGCGTAGCACAGGTGGACCAC  
 T G F F A H A G F C L E H A S C P P G A  
 841 CCGGCGTGATTGCCCCGGGCACCCCAGCCAGAACACGCAGTGCCAGCCGTGCCCCCAG 900  
 -----+-----+-----+-----+-----+-----+  
 GGCCGCACTAACGGGGCCCGTGGGGTGGTCTTGTGCGTCACGTCGGCACGGGGGGTC  
 G V I A P G T P S Q N T Q C Q P C P P G  
 901 GCACCTTCTCAGCCAGCAGCTCCAGCTCAGAGCAGTGCCAGCCCCACCGCAACTGCACGG 960  
 -----+-----+-----+-----+-----+-----+  
 CGTGGAAGAGTCGGTCGTCGAGGTCGAGTCTCGTCACGGTCGGGGTGGCGTTGACGTGCC  
 T F S A S S S S S E Q C Q P H R N C T A  
 961 CCCTGGGCTGGCCCTCAATGTGCCAGGCTCTTCTCCCATGACACCCTGTGCACCAGCT 1020  
 -----+-----+-----+-----+-----+-----+  
 GGGACCCGGACCGGGAGTTACACGGTCCGAGAAGGAGGGTACTGTGGGACACGTGGTCGA  
 L G L A L N V P G S S S H D T L C T S C  
 1021 GCACCTGGCTTCCCCCTCAGCACCAGGGTACCAGGAGCTGAGGAGTGTGAGCGTGCCGTCA 1080  
 -----+-----+-----+-----+-----+-----+  
 CGTGACCGAAGGGGGAGTCGTGGTCCCATGGTCTCGACTCCTCACACTCGCACGGCAGT  
 T G F P L S T R V P G A E E C E R A V I  
 1081 TCGACTTTGTGGCTTCCAGGACATCTCCATCAAGAGGCTGCAGCGGCTGCTGCAGGCCC 1140  
 -----+-----+-----+-----+-----+-----+  
 AGCTGAAACACCGAAAGGTCTGTAGAGGTAGTTCTCCGACGTCGCCGACGACGTCCGGG  
 D F V A F Q D I S I K R L Q R L L Q A L  
 1141 TCGAGGCCCCGGAGGGCTGGGGTCCGACACCAAGGGCGGGCCGCGCGCCTTGCAGCTGA 1200  
 -----+-----+-----+-----+-----+-----+  
 AGCTCCGGGGCTCCCGACCCAGGCTGTGGTTCGGCGGGCGCGCGCGGAACGTGCACT  
 E A P E G W G P T P R A G R A A L Q L K  
 1201 AGCTGCGTCGGCGGCTCACGGAGCTCCTGGGGGCGCAGGACGGGGCGCTGCTGGTGGGGC 1260  
 -----+-----+-----+-----+-----+-----+  
 TCGACGCAGCCCGCGAGTGCCTCGAGGACCCCGCGTCTGCCCCGCGACGACCACGCCG  
 L R R R L T E L L G A Q D G A L L V R L  
 1261 TGCTGCAGGCGCTGCCGCTGGCCAGGATGCCCGGGCTGGAGCGGAGCGTCCGTGAGCGCT 1320  
 -----+-----+-----+-----+-----+-----+  
 ACGACGTCCGCGACGCGCACCGGTCTACGGGCCGACCTCGCTCCGAGGCACTCCGGA  
 L Q A L R V A R M P G L E R S V R E R F  
 1321 TCCTCCCTGTGCACTGATCCTGGCCCCCTCTTATTTATTCTACATCCTTGGCACCCCACT 1380  
 -----+-----+-----+-----+-----+-----+  
 AGGAGGGACAGTGACTAGGACCGGGGAGAATAAATAAGATGTAGGAACCGTGGGGTGA  
 L P V H \* (SEQ ID NO:3)  
 1381 TGCAGTAAAGAGGCTTTTTTTTAAATAGAAGAAATGAGGTTTCTTAA (SEQ ID NO:1) 1428  
 -----+-----+-----+-----+-----+  
 ACGTGACTTTCTCCGAAAAAAATTTATCTTCTTTACTCCAAAGAATT (SEQ ID NO:11)

FIG. 2B

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1 MRALEGPGLS LLCLVLALPA LLPVPAVRGV AETPTYPWRD AETGERLVCA  
51 QCPPGTFVQR PCRRDSPTTC GPCPPRHYTQ FWNLYLRCRY CNVLCGEREE  
101 EARACHATHN RACRCRTGFF AHAGFCLEHA SCPPGAGVIA PGTPSQNTQC  
151 QCPPPGTFSA SSSSSEQCQP HRNCTALGLA LNVPGSSSHD TLCTSCTGFP  
201 LSTRVPGAEE CERAVIDFVA FQDISIKRLQ RLLQALEAPE GWGPTPRAGR  
251 AALQLKLRRR LTELLGAQDG ALLVRLLQAL RVARMPGLER SVRERFLPVH  
(SEQ ID NO:3)

FIG.3

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1 VAETPTYPWR DAETGERLVC AQCPGTFVQ RPCRRDSPTT CGPCPPRHYT  
51 QFWNYLERCR YCNVLCGERE EEARACHATH NRACRCRTGF FAHAGFCLEH  
101 ASCPPGAGVI APGTPSQNTQ CQPCPPGTFS ASSSSSEQCQ PHRNCTALGL  
151 ALNVPGSSSH DTLCTSCTGF PLSTRVPGAE ECERAVIDFV AFQDISIKRL  
201 QRLLQALEAP EGNQPTPRAG RAALQLKLRR RLTELLGAQD GALLVRLQLA  
251 LRVARMPGLE RSVRERFLPV H (SEQ ID NO:4)

FIG.4

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Query: 34 PTYPWRDAETGERLVCACPPGTFVQPCRRDSTTCGPCPRHYTQFWNYLERCRYCNV 93  
 P Y D E T +L+C +CPPGT+++ C T C PCP +YT W+ + C YC+

Sb jct: 26 PKYLHYDEETSHQLLCDKCPPGTYLKQHCTAKWKTVCAPCPDHYTDSWHTSDECLYCSP 64

Query: 94 LCGEREEARACHATHNRACRCRTGFFAHAGFCLEHASCPCGAGVAPGTPSQNTCCQPC 153  
 +C E + + C+ THNR C C+ G + FCL+H SCPPG GV+ GTP +NT C+ C

Sb jct: 86 VKELQYVKQECNRTHNRVCECKEGRYLEIEFCLKHRSCPPGFGVVQAGTPERNVTCKRC 124

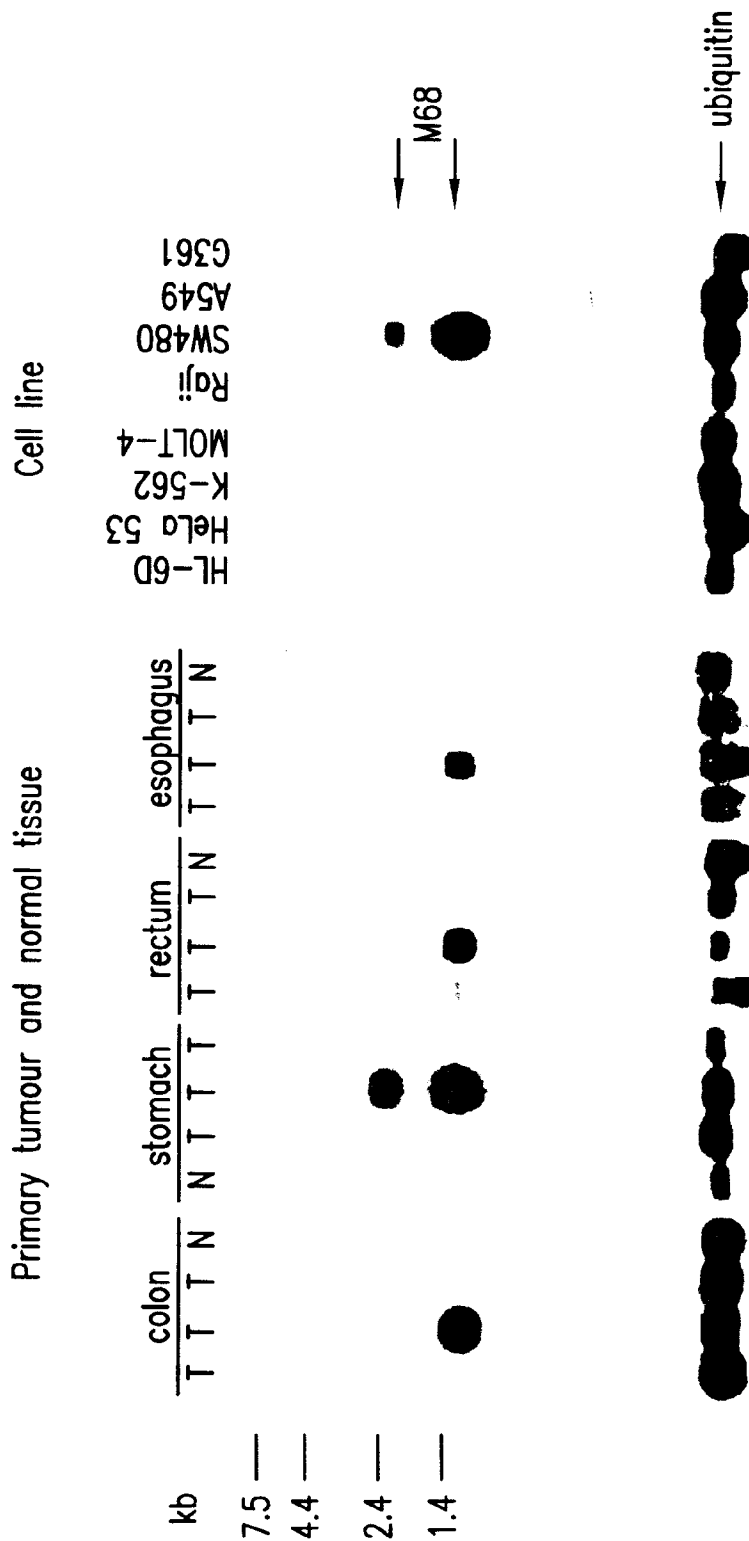
Query: 154 PPGTFSASSSSSEQQPHRNC TALGLALNVP GSSSHDILCT 194  
 P G FS +SS C+ H NC+ GL L G+++HD +C+

Sb jct: 146 PDGFFSNETSSKAPCRKHTNCSVFGLLLTQGNATHDNICS 165

Query: 211 CERAVIDFVAFQDISIKRLQRLQAL 236 (SEQ ID NO:3)  
 CE +V + +++ ++L+ L+++L

Sb jct: 277 CENSQVRHIGHANLTFEQLRSLMESL 281 (SEQ ID NO:10)

FIG.5



**FIG. 6A**

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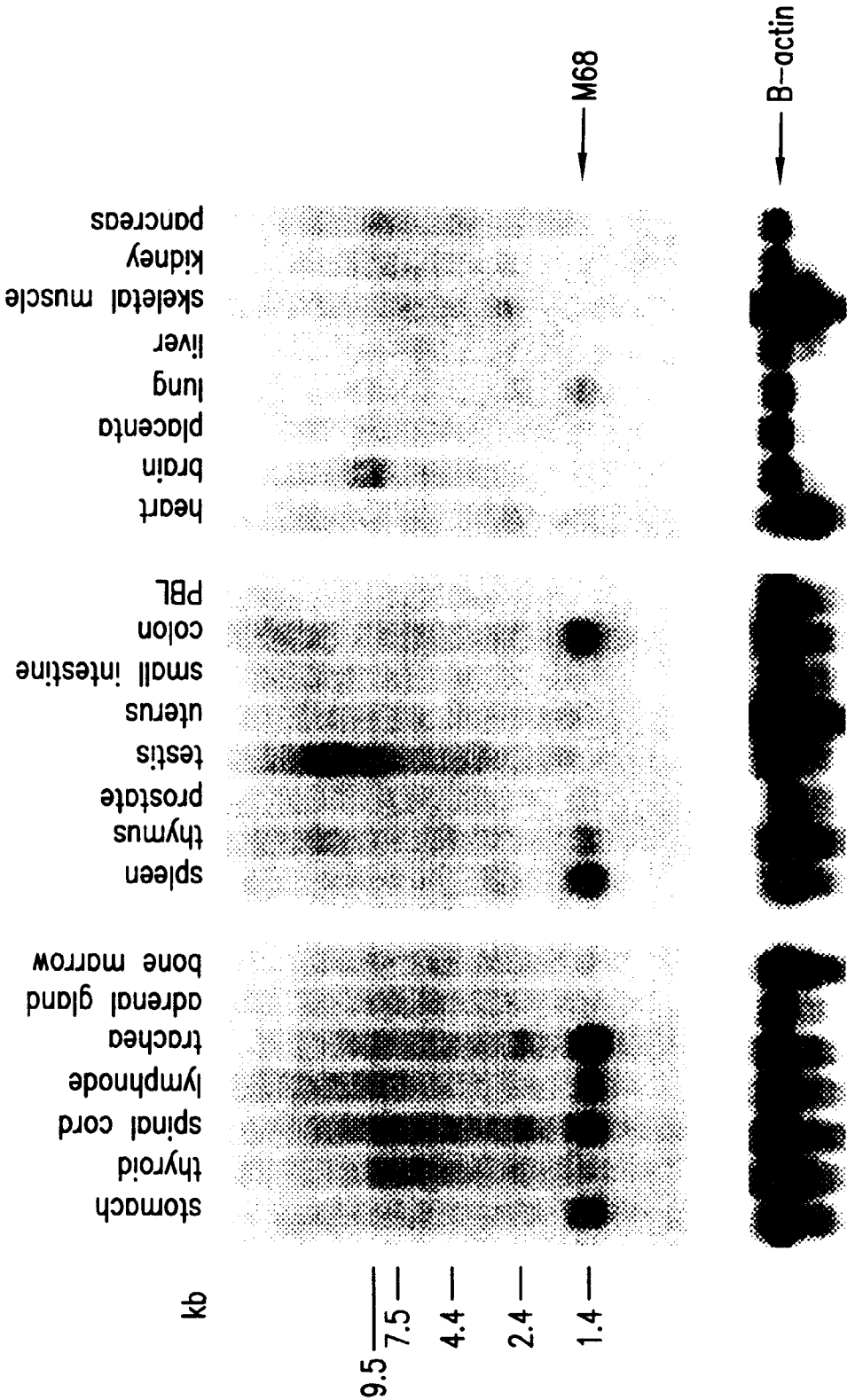


FIG. 6B

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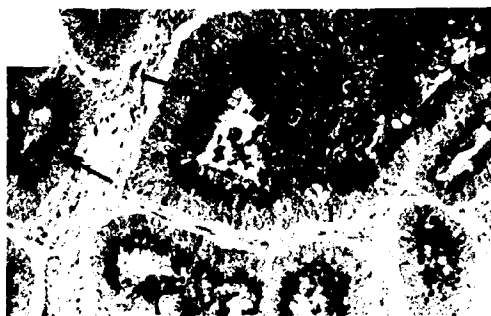


FIG. 7A

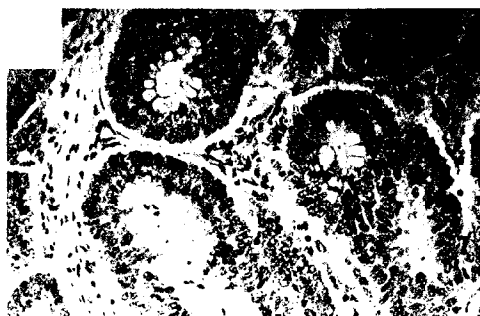


FIG. 7B



FIG. 7C

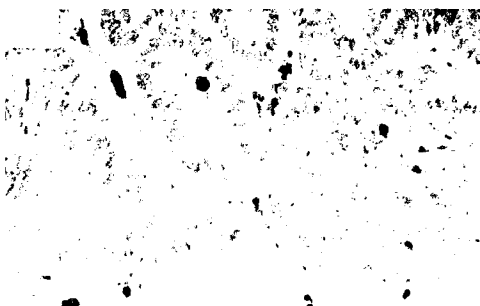


FIG. 7D

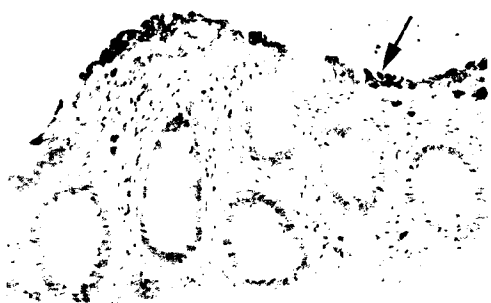


FIG. 7E

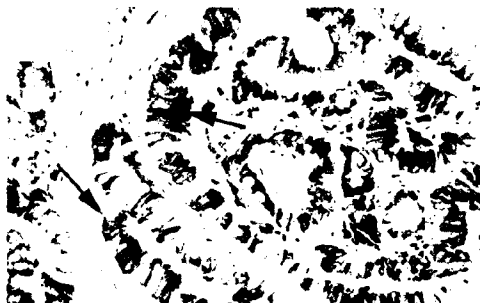


FIG. 7F

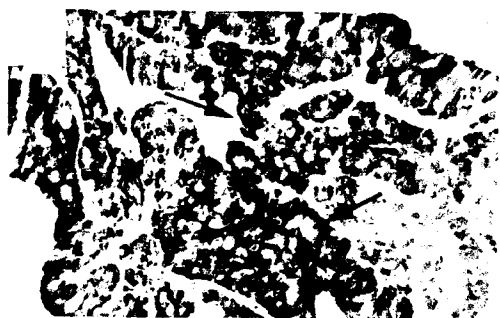


FIG. 7G

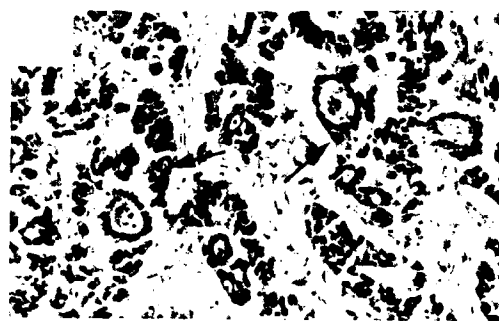


FIG. 7H

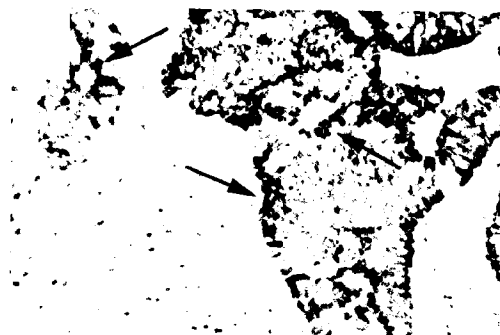


FIG. 7I



FIG. 7J

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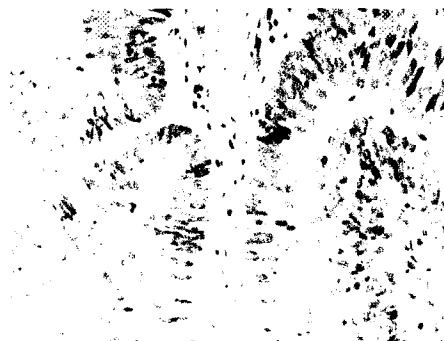


FIG. 8A



FIG. 8B

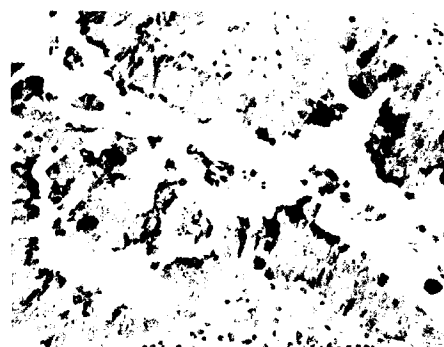


FIG. 8C

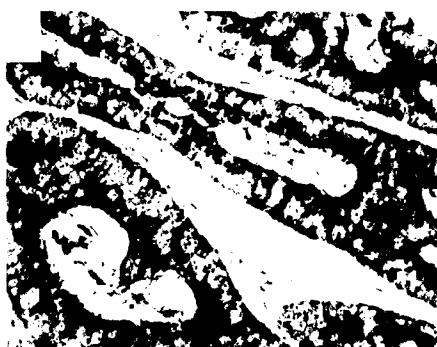


FIG. 8D

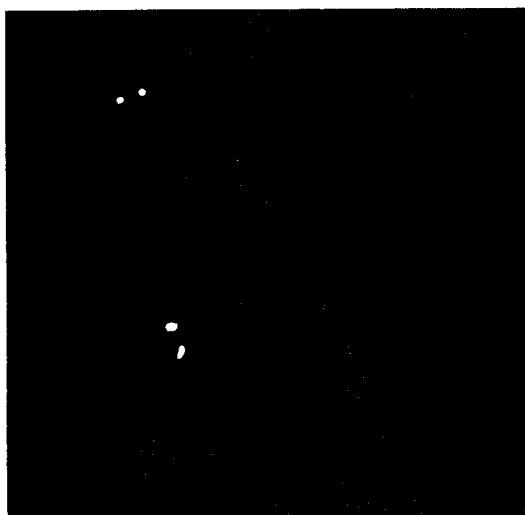


FIG. 9B



FIG. 9D

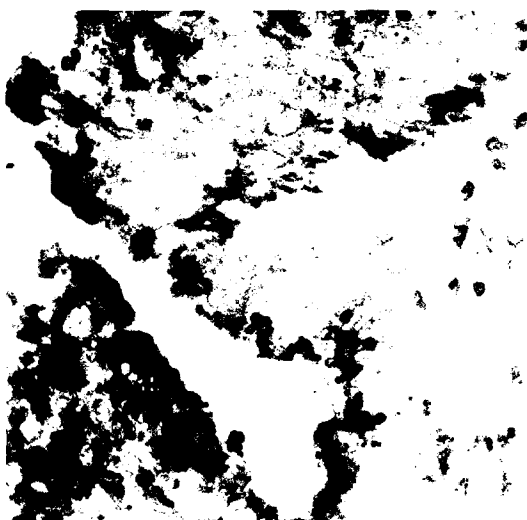


FIG. 9A

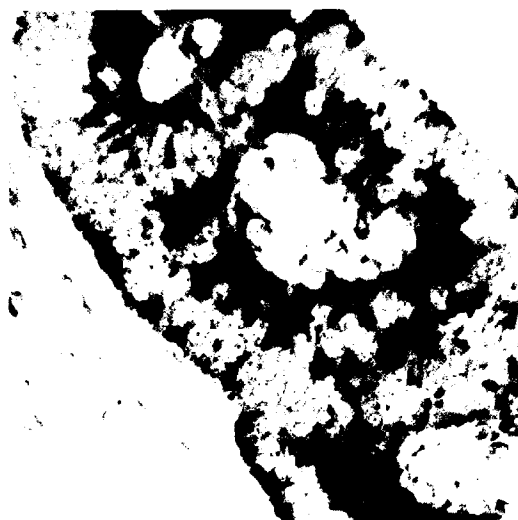


FIG. 9C

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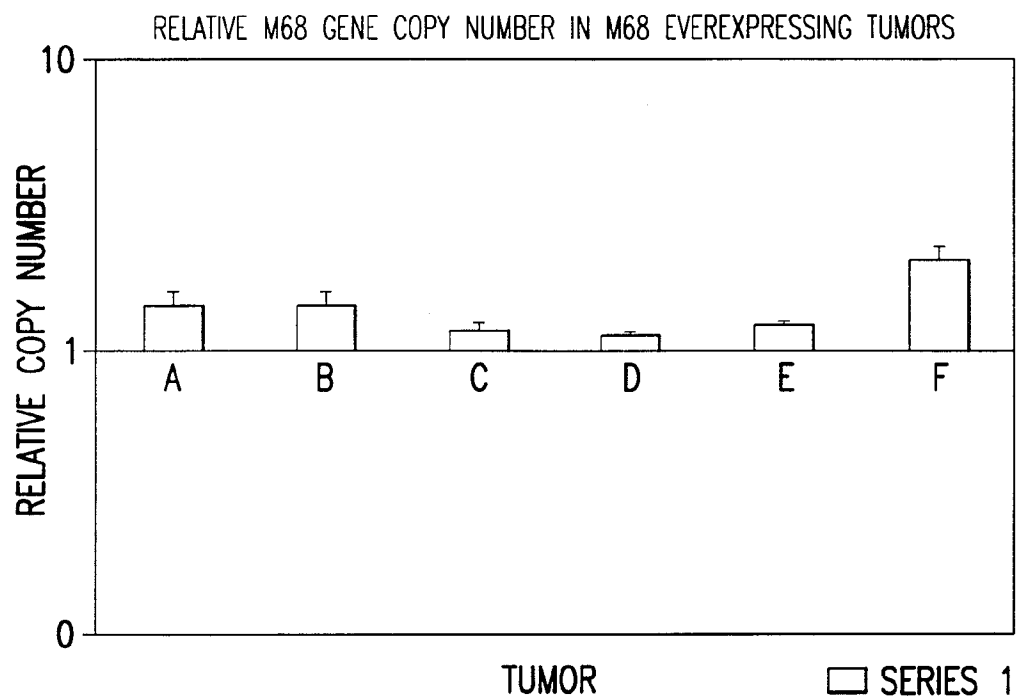


FIG. 9E

## SEQUENCE LISTING

<110> APPLICANT: Merck & Co., Inc (CA, EP, JP)  
Bai, Chang (US only)

<120> TITLE: METHODS OF USE FOR DNA MOLECULES  
ENCODING M68, A TNF RECEPTOR-RELATED PROTEIN

<130> DOCKET/FILE REFERENCE: 20392Y

<160> NUMBER OF SEQUENCES: 21

<170> SOFTWARE: FastSEQ for Windows Version 4.0

<210> SEQ ID NO:1

<211> LENGTH: 1428

<212> TYPE: DNA

<213> ORGANISM:Homo sapien

<400> SEQ ID NO:1

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ggcatggagc	caccgggacc	ccaggaggag	aggcttgccg	tgccctcctgt	gcttaccac	180
agggctcccc	aaccaggccc	ctcacggctc	gagaagaccg	ggaagaccca	gagcaagatc	240
tcgtcccttc	ttagacagag	gccagcaggg	actgtggggg	cgggcgggtga	ggatgcagggt	300
cccagccagt	cctcaggacc	tccccacggg	cctgcagcat	ctgagtgggg	cctctaggat	360
gtgcccagcc	tgccacaccg	cctccaggaa	gcagagcgtc	atgcaggtct	tctggccgga	420
gccccacaag	gaccatgagg	gcgctggagg	ggccaggcct	gtcgtctgtg	tgcttggtgt	480
tggtcgctgc	tgccctgctg	ccggtgccgg	ctgtacgcgg	agtggcagaa	acacccacct	540
acccctggcg	ggacgcagag	acaggggagc	ggctggtgtg	cggccagtgc	ccccaggca	600
cctttgtgca	gcggccgtgc	cggcgagaca	gccccacgac	gtgtggcccc	tgtccaccgc	660
gccactacac	gcagttctgg	aactacctgg	agcgtctgcc	ctactgcaac	gtcctctgcg	720
gggagcgtga	ggaggaggca	cgggcttgcc	acgccaccca	caaccgtgcc	tgccgctgcc	780
gcaccggcct	cttcgcgcac	gctgggtttc	gcttgagca	cgcctcgtgt	ccacctggtg	840
ccggcgctgat	tgccccgggc	acccccagcc	agaacacgca	gtgccagccg	tgccccccag	900
gcaccttctc	agccagcagc	tccagctcag	agcagtgcc	gccccaccgc	aactgcacgg	960
ccctgggcct	ggccctcaat	gtgccaggct	cttcctccca	tgacaccttg	tgccaccagt	1020
gcactggcct	ccccctcagc	accagggtac	caggagctga	ggagtgtgag	cgtgccgtca	1080
tcgactttgt	ggctttccag	gacatctcca	tcaagaggct	gcagcggctg	ctgcaggccc	1140
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tcctccctgt	gcactgatec	tggccccctc	ttatttatcc	tacatccttg	gcacccact	1380
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<222> LOCATION: (53)...(955)

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Ala Leu Glu Gly Pro Gly Leu Ser Leu Leu Cys Leu Val Leu Ala Leu	
5 10 15	
cct gcc ctg ctg ccg gtg ccg gct gta cgc gga gtg gca gaa aca ccc	154
Pro Ala Leu Leu Pro Val Pro Ala Val Arg Gly Val Ala Glu Thr Pro	
20 25 30	
acc tac ccc tgg cgg gac gca gag aca ggg gag cgg ctg gtg tgc gcc	202
Thr Tyr Pro Trp Arg Asp Ala Glu Thr Gly Glu Arg Leu Val Cys Ala	
35 40 45 50	
cag tgc ccc cca ggc acc ttt gtg cag cgg ccg tgc cgc cga gac agc	250
Gln Cys Pro Pro Gly Thr Phe Val Gln Arg Pro Cys Arg Arg Asp Ser	
55 60 65	
ccc acg acg tgt ggc ccg tgt cca ccg cgc cac tac acg cag ttc tgg	298
Pro Thr Thr Cys Gly Pro Cys Pro Pro Arg His Tyr Thr Gln Phe Trp	
70 75 80	
aac tac ctg gag cgc tgc cgc tac tgc aac gtc ctc tgc ggg gag cgt	346
Asn Tyr Leu Glu Arg Cys Arg Tyr Cys Asn Val Leu Cys Gly Glu Arg	
85 90 95	
gag gag gag gca cgg gct tgc cac gcc acc cac aac cgt gcc tgc cgc	394
Glu Glu Glu Ala Arg Ala Cys His Ala Thr His Asn Arg Ala Cys Arg	
100 105 110	
tgc cgc acc ggc ttc ttc gcg cac gct ggt ttc tgc ttg gag cac gca	442
Cys Arg Thr Gly Phe Phe Ala His Ala Gly Phe Cys Leu Glu His Ala	
115 120 125 130	
tcg tgt cca cct ggt gcc ggc gtg att gcc ccg ggc acc ccc agc cag	490
Ser Cys Pro Pro Gly Ala Gly Val Ile Ala Pro Gly Thr Pro Ser Gln	
135 140 145	
aac acg cag tgc cag ccg tgc ccc cca ggc acc ttc tca gcc agc agc	538
Asn Thr Gln Cys Gln Pro Cys Pro Pro Gly Thr Phe Ser Ala Ser Ser	
150 155 160	
tcc agc tca gag cag tgc cag ccc cac cgc aac tgc acg gcc ctg ggc	586
Ser Ser Ser Glu Gln Cys Gln Pro His Arg Asn Cys Thr Ala Leu Gly	
165 170 175	
ctg gcc ctc aat gtg cca ggc tct tcc tcc cat gac acc ctg tgc acc	634
Leu Ala Leu Asn Val Pro Gly Ser Ser Ser His Asp Thr Leu Cys Thr	
180 185 190	
agc tgc act ggc ttc ccc ctc agc acc agg gta cca gga gct gag gag	682
Ser Cys Thr Gly Phe Pro Leu Ser Thr Arg Val Pro Gly Ala Glu Glu	
195 200 205 210	
tgt gag cgt gcc gtc atc gac ttt gtg gct ttc cag gac atc tcc atc	730
Cys Glu Arg Ala Val Ile Asp Phe Val Ala Phe Gln Asp Ile Ser Ile	
215 220 225	
aag agg ctg cag cgg ctg ctg cag gcc ctc gag gcc ccg gag ggc tgg	778
Lys Arg Leu Gln Arg Leu Leu Gln Ala Leu Glu Ala Pro Glu Gly Trp	
230 235 240	

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ggt ccg aca cca agg gcg ggc cgc gcg gcc ttg cag ctg aag ctg cgt      826
Gly Pro Thr Pro Arg Ala Gly Arg Ala Ala Leu Gln Leu Lys Leu Arg
      245                      250                      255

cgg cgg ctc acg gag ctc ctg ggg gcg cag gac ggg gcg ctg ctg gtg      874
Arg Arg Leu Thr Glu Leu Leu Gly Ala Gln Asp Gly Ala Leu Leu Val
      260                      265                      270

cgg ctg ctg cag gcg ctg cgc gtg gcc agg atg ccc ggg ctg gag cgg      922
Arg Leu Leu Gln Ala Leu Arg Val Ala Arg Met Pro Gly Leu Glu Arg
      275                      280                      285                      290

agc gtc cgt gag cgc ttc ctc cct gtg cac tga tcctggcccc ctcttattta      975
Ser Val Arg Glu Arg Phe Leu Pro Val His *
      295                      300

ttctacatcc ttggcacccc acttgcaactg aaagaggctt tttttttaaata agaagaaatg      1035
aggtttctta aaaaaaaaaa aaaaaaaaaa a
      1066

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&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM:Homo sapien

&lt;400&gt; SEQ ID NO:3

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Ala Leu Pro Ala Leu Leu Pro Val Pro Ala Val Arg Gly Val Ala Glu
      20          25          30
Thr Pro Thr Tyr Pro Trp Arg Asp Ala Glu Thr Gly Glu Arg Leu Val
      35          40          45
Cys Ala Gln Cys Pro Pro Gly Thr Phe Val Gln Arg Pro Cys Arg Arg
      50          55          60
Asp Ser Pro Thr Thr Cys Gly Pro Cys Pro Pro Arg His Tyr Thr Gln
      65          70          75          80
Phe Trp Asn Tyr Leu Glu Arg Cys Arg Tyr Cys Asn Val Leu Cys Gly
      85          90          95
Glu Arg Glu Glu Glu Ala Arg Ala Cys His Ala Thr His Asn Arg Ala
      100          105          110
Cys Arg Cys Arg Thr Gly Phe Phe Ala His Ala Gly Phe Cys Leu Glu
      115          120          125
His Ala Ser Cys Pro Pro Gly Ala Gly Val Ile Ala Pro Gly Thr Pro
      130          135          140
Ser Gln Asn Thr Gln Cys Gln Pro Cys Pro Pro Gly Thr Phe Ser Ala
      145          150          155          160
Ser Ser Ser Ser Ser Glu Gln Cys Gln Pro His Arg Asn Cys Thr Ala
      165          170          175
Leu Gly Leu Ala Leu Asn Val Pro Gly Ser Ser Ser His Asp Thr Leu
      180          185          190
Cys Thr Ser Cys Thr Gly Phe Pro Leu Ser Thr Arg Val Pro Gly Ala
      195          200          205
Glu Glu Cys Glu Arg Ala Val Ile Asp Phe Val Ala Phe Gln Asp Ile
      210          215          220
Ser Ile Lys Arg Leu Gln Arg Leu Leu Gln Ala Leu Glu Ala Pro Glu
      225          230          235          240
Gly Trp Gly Pro Thr Pro Arg Ala Gly Arg Ala Ala Leu Gln Leu Lys
      245          250          255
Leu Arg Arg Arg Leu Thr Glu Leu Leu Gly Ala Gln Asp Gly Ala Leu
      260          265          270

```

Leu Val Arg Leu Leu Gln Ala Leu Arg Val Ala Arg Met Pro Gly Leu  
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 Glu Arg Ser Val Arg Glu Arg Phe Leu Pro Val His  
                   290                                  295                                  300

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 <213> ORGANISM:Homo sapien

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 Asp Thr Leu Cys Thr Ser Cys Thr Gly Phe Pro Leu Ser Thr Arg Val  
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 Pro Gly Ala Glu Glu Cys Glu Arg Ala Val Ile Asp Phe Val Ala Phe  
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 Gln Asp Ile Ser Ile Lys Arg Leu Gln Arg Leu Leu Gln Ala Leu Glu  
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 <213> ORGANISM:Artificial Sequence

<220> FEATURE:  
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<210> SEQ ID NO:7  
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<210> SEQ ID NO:8  
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 <213> ORGANISM:Artificial Sequence

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<400> SEQ ID NO:8  
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<210> SEQ ID NO:9  
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<210> SEQ ID NO:10  
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 Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr Tyr Thr Asp Ser  
 35 40 45  
 Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro Val Cys Lys Glu  
 50 55 60  
 Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg Val Cys  
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Arg	Ser	Cys	Pro	Pro	Gly	Phe	Gly	Val	Val	Gln	Ala	Gly	Thr	Pro	Glu
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<210> SEQ ID NO:12
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM:Homo sapien
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Val His
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<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM:Homo sapien
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<400> SEQ ID NO:13

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<210> SEQ ID NO:14

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<212> TYPE: PRT

<213> ORGANISM:Homo sapien

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<210> SEQ ID NO:15

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM:Homo sapien

<400> SEQ ID NO:15

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<210> SEQ ID NO:16

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<212> TYPE: DNA

<213> ORGANISM:Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotide

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<210> SEQ ID NO:17

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<212> TYPE: DNA

<213> ORGANISM:Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotide

<400> SEQ ID NO:17

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17

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<213> ORGANISM:Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: oligonucleotide

<400> SEQ ID NO:18

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<210> SEQ ID NO:19  
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<210> SEQ ID NO:20  
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<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotide

<400> SEQ ID NO:20  
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<210> SEQ ID NO:21  
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<213> ORGANISM:Artificial Sequence

<220> FEATURE:  
<223> OTHER INFORMATION: oligonucleotide

<400> SEQ ID NO:21  
ctacccttgg acccagaggt tctttgagtc 30

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/03037

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : Please See Extra Sheet.

US CL : 530/ 350; 536/23.5; 435/6, 7.1, 320.1, 361; 514/2, 44

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/ 350; 536/23.5; 435/6, 7.1, 320.1, 361; 514/2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Commercial Sequence Databases, MPSRCH.

sequences searched: SEQ ID NOS: 1-5

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 98/30694 A2 (HUMAN GENOME SCIENCES, INC.) 16 July 1998 (16.07.98), see entire document, especially Fig. 1, Claims 2-4, 19-21, 22 and 23, and pages 31-35, page 39, line 36 to page 40, line 5, page 40, lines 13-30, page 41, lines 5-15, page 41 line 19 to page 44, line 3.	1, 3, 6, 9, 14-18, 21, 22, 25-30 ----- 2, 4, 5, 7, 8, 10-13, 19, 20, 23
X -- Y	WO 99/04001 A1 (ZYMOGENETICS, INC.) 28 January 1999 (28.01.99), see entire document, especially SEQ ID NOS: 1 and 2, claims 1-3, 5, 11, 21 and 22, and page 51, lines 21 to page 52, line 18, page 45, lines 23-35, page 48, lines 1-7, page 49, line 29 to page 50, line 26.	1, 9, 14-18, 21, 22, 25-30 ----- 2-8, 10-13, 19, 20, 23



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & * document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 MAY 2000

Date of mailing of the international search report

07 JUL 2000

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/03037

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	EP 0 861 850 A1 (SMITHKLINE BEECHAM CORPORATION) 02 September 1998 (02.09.98), see entire document, especially Fig. 1, claims 14-18, 21, 22, 24, and pages 9-10.	1, 3, 9, 14-18, 25-30 ----- 2, 4-8, 10-13, 19, 20-23
X	PITTI, ROBERT M. Genomic Amplification of a Decoy Receptor for Fas Ligand in Lung and Colon Cancer. Nature. 17 December 1998, Vol. 396, pages 699-702, especially Figure 1a. Also see attached sequence alignment.	21, 22, 25-28
Y	Database EST, National Cancer Institute, Cancer Genome Project (CGAP), Tumor Gene Index, AN AI304479, qo54d03.x1 NCI_cGAP_Co8 Homo Sapiens cDNA Clone IMAGE:1912325 3' similar to contains LTR5.b1 MER22 Repetitive Element; mRNA sequence. 01 February 1999, see alignment with SEQ ID NO:5.	23

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/03037

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/03037

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

C07K 14/47; C07H 21/04; C12N 15/62, 15/63, 5/22; G01N 33/52, 33/53; A61K 31/70, 38/17

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-7 and 19-28, drawn to nucleic acids encoding M68 protein, M68 protein, and a method for determining whether a substance is capable modulating M68 protein using cells transformed with M68 nucleic acids.

Group II, claim(s) 8, drawn to a method of determining whether a substance is a potential modulator of M68 gene expression.

Group III, claim(s) 9-13, drawn to a method of inhibiting Fas-mediated apoptosis by administering nucleic acids encoding M68 protein.

Group IV, claim(s) 14-18, drawn to a method of inhibiting Fas-mediated apoptosis by administering M68 protein.

Group V, claim(s) 29, drawn to an antibody that binds M68 protein.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Pursuant to 37 C.F.R. § 1.475(d), the ISA/US considers that where multiple products and processes are claimed, the main invention shall consist of the first product claimed. Accordingly, the main invention (Group I) corresponds to the first product mentioned which is nucleic acid molecules encoding M68 protein, the first method of using the nucleic acid molecules which is a method of determining whether a substance is a potential modulator of M68 protein using cells transformed with the nucleic acids, and the protein encoded by the nucleic acid molecules. Further pursuant to 37 C.F.R. § 1.475(d), the ISA/US considers that any feature which the subsequently recited products and methods share with the main invention does not constitute a special technical feature within the meaning of PCT Rule 13.2 and that each of such products and methods accordingly defines a separate invention.